Studies on Expression of γ-Glutamyltranspeptidase of Escherichia Coli K-12(Dissertation_全文)

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List of Abbreviations

ECL	enhanced chemiluminescence
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
GGT	γ-glutamyltranspeptidase
γ-Glu-*p*NA	L-γ-glutamyl-*p*-nitroanilide
Glygly	glycylglycine
HPLC	high performance liquid chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
M-MLV	moloney murine leukemia virus
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PIPES	piperazine *N*, *N'*-bis(2-ethanesulfonic acid)
PNPase	polynucleotide phosphorylase
PVDF	polyvinylidene difluoride
P1 *vir*	P1 virulent phage
RNase	ribonuclease
SDS	sodium dodecyl sulfate
S. typhimurium	*Salmonella typhimurium*

TBS	tris buffered saline
Tris	tris(hydroxymethyl)aminomethane
U	units

Introduction

γ-Glutamyltranspeptidase (GGT: EC 2.3.2.2), which catalyzes the hydrolysis of γ-glutamyl compounds such as glutathione, and the transfer of their γ-glutamyl moieties to amino acids and peptides, is widely distributed in living organisms and is a key enzyme in glutathione metabolism (1). GGT of *Escherichia coli* K-12 is a periplasmic enzyme, composed of one large subunit and one small subunit (2, 3). The nucleotide sequence of its gene indicates that the gene codes a signal peptide, large subunit, and small subunit in a single open reading frame in this order (4). Therefore, pre-pro-GGT must undergo two proteolytic processing steps to generate its catalytically active dimeric form in the periplasm. This kind of processing mechanism is very rare in *E. coli*, other examples being penicillin-G acylase (5) and S-adenosylmethionine decarboxylase (6). The processing mechanism has been studied in mammalian GGT (7), while bacterial GGTs have not been elucidated in respect to processing. Moreover among all GGTs, the importance of the amino acid residues at the cleavage site is not understood.

Although heat shock proteins in *E. coli* were well studied, it was not until recently that cold shock and/or inducible proteins have been actively examined (8, 9, 10). Since GGT activity in *E. coli* K-12 cells grown at 20℃ was 7-fold higher than that grown at an optimal growth temperature (2), the

GGT of *E. coli* K-12 may be a cold shock protein.

The catalytic center of GGTs has been studied using chemical modification and site-directed mutagenesis. Treatments of mammalian GGTs with chemical modifying agents for arginyl residues were reported to inactivate them (11, 12, 13), and an arginyl residue in the small subunit has been proposed to be involved in the recognition of an anionic moiety of an acceptor in the transfer reaction by way of an electrostatic interaction, and arginyl residue-111 of rat GGT has been reported to interact glutathione. Arginyl residue-107 of human GGT has been reported to play a significant role in binding the substrate, by site-directed mutagenesis (14).

Three subsites have been identified in the active center of GGT (1), one was the γ-glutamyl donor site and the others were acceptor sites. The donor site is assumed to be in the small subunit, and both large and small subunits to participate in formation of the acceptor site. However, both subunits of hepatoma GGT have been reported to possess enzymatic activity, using the separated subunits in a reversed micelle system (15), and that those of *E. coli* GGT could be separated by high-performance liquid chromatography and neither subunit alone exhibited the enzymatic activity (16).

In this thesis, the author studied the expression of *E. coli* K-12 GGT protein and its activity to elucidate the expression induced at a low temperature, the maturation mechanism, the possible active center, and the interaction of its

interaction of its subunits.

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Chapter 1. Low Temperature Inducible γ-Glutamyltranspeptidase of *Escherichia coli* K-12

γ-Glutamyltranspeptidase (GGT: EC 2.3.2.2) catalyzes the hydrolysis of γ-glutamyl compounds such as glutathione, and the transfer of their γ-glutamyl moieties to amino acids and peptides (1). GGT is widely distributed in living organisms and some have been cloned (2, 3, 4, 5, 6, 7). GGT has been designated as the key enzyme in glutathione metabolism and we showed that GGT is essential for the assimilation of exogenous γ-glutamyl peptides as an amino acid source in *Escherichia coli* K-12 cells (8). GGT of *E. coli* K-12 is localized in the periplasm (9), and consists of one large subunit and one small subunit (10). The nucleotide sequence of its gene indicates that a signal peptide, the large subunit and the small subunit are encoded in a single open reading frame in this order (11). Therefore, the heterodimer is thought to be synthesized from a single precursor polypeptide by post-translational processing. The process of proteolytic cleavage is suggested to be important for correct folding of the active form by site-directed mutagenesis (12, 13, also chapter 2, 3).

E. coli K-12 cells exhibit maximum GGT activity when grown at 20 ℃, 14% of that at 37℃ and GGT of *E. coli* K-12 is stable in the cells at higher temperature (9). In *E. coli*, there are several proteins expressed by cold shock

and some of them are identified (14). In *E. coli*, major cold shock protein (CS7.4) (15) is thought to regulate the expression of the cold shock genes (16). CS7.4 is reported to have high similarity to human DNA binding proteins and it binds to the consensus sequence, 5'-CCAAT-3', and its contemporary sequence, 5'-ATTGG-3' (17). Some promoters of *E. coli* have been identified as being low-temperature inducible, using a fusion gene composed of the transposon gene and *lacZ* (18).

In this chapter, the expression of GGT activity in *E. coli* K-12 cells grown at low temperature was analyzed, the promoter region of *ggt* was identified and characterized, and a new strain, which produced more GGT at 37℃, was constructed.

MATERIALS AND METHODS

Materials. Restriction endonucleases, other DNA modification enzymes, sequencing primers, and polymerase chain reaction (PCR) primers were purchased from Takara Shuzo Co., Toyobo Co., and Nippon Gene Co. M-MLV reverse transcriptase was purchased from GIBCO BRL LIFE TECHNOLOGIES, INC. Ribonuclease inhibitor was purchased from Promega Co. Nylon membrane (Hybond N+), anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody (from donkey), and ECL

Western blotting detection reagents were purchased from Amersham International plc. X-ray films were purchased from Fuji Photo Film Co. Polyvinylidene difluoride (PVDF) membranes were purchased from Nihon Millipore Kogyo K.K.

Bacterial strains. The bacterial strains used in this chapter are listed in Table 1-1.

Table 1-1. Bacterial strains used in this chapter.

Strain	Genotype	Source and reference
Escherichia coli K-12		
MG1655	Prototrophic	(19)
MC1061 *recA*	*△(lacIPOZYA)X74 galU galK* $strA^R$ *△(ara-leu) hsdR⁻ recA*	(20)
SH641	F⁻ *ggt-2 recA56 rpsL srl300*::Tn*10*	(4)
Escherichia coli B		
355	Wild type	Murata (21)
Salmonella typhimurium		
AKU0094	Wild type	Kobayashi and Shimizu

Growth media. LB medium (22) without or with appropriate antibiotics at 30~100 μg/ml was used for preculturing of the strains harboring recombinant plasmids. Overnight preculture was subcultured at a dilution of 1:100 in LB medium and grown at 20℃ or at 37℃.

Protein concentration and GGT activity determination. The protein concentration was determined by the method of Lowry *et al.* (23), with bovine serum albumin, as a standard. GGT activity was determined with L-γ-glutamyl-*p*-nitroanilide (γ-Glu-*p*NA) as a substrate and glycylglycine (Glygly) as an acceptor (9). The assay solution contained 0.25μmol of γ-Glu-*p*NA, 30μmol of Glygly, 25μmol of Tris-HCl (pH8.73) and the enzyme in a final volume of 0.5ml. After incubation at 37℃ for 15 min, the reaction was terminated by the addition of 1ml of 3.5N acetic acid and the absorbance at 410nm was measured.

Subcloning, transformation, and gel electrophoresis. Subcloning, transformation, and gel electrophoresis were performed as described (24, 25).

DNA sequence. The correctness of the DNA sequences of the mutant plasmids was confirmed by the dideoxy-chain termination method (26, 27) with BcaBest Dideoxy Sequencing Kit (Takara Shuzo Co.), using [α-^{32}P]dCTP (ICN Biomedicals Inc.).

Preparation of periplasmic fraction. The periplasmic fraction was obtained by lysozyme treatment, as described (9).

Preparation of cell free extracts. For the β-galactosidase assay, cells cultured until the absorbance at 600nm equaled about 1.0, were harvested by centrifugation 4,000 x g for 10min. After two washes with 50mM sodium phosphate buffer pH7.0, the cells were resuspended in the same buffer. The

cells were disrupted by ultrasonic treatment and the supernatant (cell free extracts) was obtained by centrifugation (15,000 x g for 20min). For Western blot analysis, cells cultured up to the stationary phase were harvested by centrifugation 11,000 x g for 5min, then lysed with lysis buffer consisting of 100mM Tris-HCl (pH6.8), 200mM dithiothreitol, 4% sodium dodecyl sulfate, 0.2% bromophenol-blue and 20% glycerol.

Electrophoresis and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described (28). Proteins on an SDS-polyacrylamide gel were electroblotted onto a PVDF membrane (29). After the PVDF membranes had been blocked with TBS-T (Tris-buffered saline, pH7.6, and 0.1% Tween 20) containing 5% skim milk, for 60min at room temperature, they were incubated with anti-*E. coli* GGT antibody (from rabbit) for 60min at room temperature. After the membranes had been washed with TBS-T, they were incubated with anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody (from donkey), for 20min at room temperature. After the membranes had been washed with TBS-T, *E. coli* GGT was detected with ECL Western blotting detection reagents according to the protocol recommended by the manufacture.

Determination of the expression level of GGT protein on Western blot analysis. The Western blot profiles were read with a scanner (Epson Co., GT6000), and densities of these protein bands were quantified with a

Macintosh Centris 650 computer (Apple Computer, Inc.) using an NIH Image 1.54 Program.

Isolation of RNA. RNA from cultured cells was isolated with phenol-SDS method, as described previously (30) with a slight modification. Overnight preculture strain MG1655 (0.2ml) was subcultured in 20ml of LB medium and grown at 20, 37, and 42℃ until an absorbance at 600nm was 0.5, respectively. Immediately, cultured cells were chilled on ice, transferred to 50ml-volume polypropylene tubes, and harvested by centrifugation at 3,000 x g for 10min. The cells were suspended by pipetting in 0.5ml of solution A consisting of 0.5% SDS, 20mM sodium acetate, and 10mM EDTA pH5.5. Saturated phenol (0.5ml) with 20mM sodium acetate and 10mM EDTA pH5.5 was added immediately to the suspension and it was mixed well at 60℃ for 5min. The mixture was transferred to 1.5ml-volume microtube and centrifuged at 11,000 x g for 3min. The water layer (about 400μl) was transferred to a new tube and 2.5-fold volumes of ethanol was added. Precipitation by ethanol was obtained by centrifugation at 11,000 x g for 5min. The pellet was washed with 1ml of 70% ethanol and resuspended in 0.4ml of solution A. This ethanol precipitation was repeated three times. The pellet (total RNA) was dried under vacuum and dissolved in 0.2ml of solution A or sterilized distilled water.

Measurement of nucleic acid. DNA and RNA were quantified by

measuring the absorbance at 260nm.

Northern blot analysis. Total RNA (30μg) from strain MG1655 was denatured at 55℃ for 15min in the presence of formaldehyde and formamide, subjected to 1% agarose gel electrophoresis in the presence of 2.2M formaldehyde and then transferred to a nylon membrane (Hybond N+) by capillary transfer in 20 x SSPE (150mM NaCl, 10mM sodium phosphate buffer, and 1mM EDTA, pH7.4). After the RNA was fixed on membrane with 50mM NaOH, the membrane was washed with 2 x SSPE and prehybridized at 42℃ for 24hr in 50% formamide containing 5 x SSPE, 5 x Denhardt's reagent (0.02% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll 400), and 20μg/ml of sermon sperm DNA. The DNA probe (0.8kb), which was a restriction fragment of *ggt* digested with *Eco*RV and *Eco*RI encoding N-terminal of GGT, was labeled in the presence of random primers, [α-^{32}P]dCTP (ICN Biomedicals Inc.), dNTP, and Klenow fragment. Hybridization was carried out for 24hr under the same condition as prehybridization in the presence of the DNA probe. After hybridization, the membrane was washed in washing buffer (2 x SSPE, 0.1% SDS) at 42℃ for 45min, washed in another washing buffer (1 x SSPE, 0.1% SDS) at 42℃ for 15min with constant agitation, air-dried, and then exposed to autoradiography with X-ray film at -70℃. The relative intensities of bands on the autoradiogram were integrated with a densitometer (Shimadzu Co., CS-930).

Primer extension analysis. Total RNA was isolated from strains SH642 (4), which produced much GGT, and MG1655 by phenol-SDS method as described above. A synthetic oligonucleotide, 5'-GGCGGCGCTAAAACAACTTCCTGAGAGCAGAGCAGC-3', specific to nucleotide positions 37 to 72 from the first base of translation initiation codons, was used as the extension primer. The primer was labeled with [γ-^{32}P]ATP (ICN Biomedicals Inc.), using T4 polynucleotide kinase. Unincorporated [γ-^{32}P]ATP was removed using Quick Spin Column Sephadex G-25 (Boehringer Mannheim Biochemica) and about 1 x 10^5cpm primer was obtained. The labeled primer was incubated with 50μg of total RNA for 10min at 85℃, annealed for 11hr at 30℃ in 40mM PIPES pH6.4, 1mM EDTA pH8.0, 0.4M NaCl, 80% formamide, then precipitated with ethanol. The annealed sample was reverse transcribed by incubating with 200units of M-MLV reverse transcriptase for 2hr at 37℃ in 50mM Tris-HCl pH8.3, 75mM KCl, 3mM $MgCl_2$, 20mM dithiothreitol, 100mM each of dNTP, and 40units of ribonuclease inhibitor. The reaction mixture was incubated with 5ng of ribonuclease A for 30min at 37℃, precipitated with ethanol, resuspended in the same formamide dye as sequencing, and analyzed on a sequencing gel.

Polymerase chain reaction (PCR). PCR reaction mixtures contained 10~100ng of template DNA, 20pmol of primers and 2units of *Taq* DNA polymerase in 100μl of PCR buffer consisting of 20mM Tris-HCl (pH8.3),

1.5mM $MgCl_2$, 25mM KCl, 100μg/ml bovine serum albumin and 50μM dNTP. Each of total 30 cycles of PCR consisted of incubation for 30sec at 95 ℃ for denaturation, 30sec at 60℃ for annealing, and 90sec at 72℃ for elongation.

Construction of plasmid pHW254. Plasmid pSH253 (31) was cleaved with *Eco*RV. The smaller *Eco*RV fragment (1.2kb) was ligated with the largest fragment (4.1kb) of pSH253 cleaved with *Sma*I and *Eco*RV. The resultant plasmid pHW254 (5.3kb) were deleted *Sma*I-*Eco*RV flanking 5'-region of *ggt* from pSH253 and *ggt* was located downstream of the first *Eco*RV site (Fig. 1-1) in pUC119 (32).

Construction of deletion mutant plasmids of 5'-region of *ggt*. To identify the promoter region of *ggt*, deletion mutants of 5'-untranslated region of *ggt* were constructed as follows. Eight oligonucleotides (Fig. 1-1, Table 1-2) that annealed to the upstream region of *ggt* and had an *Eco*RI site, were synthesized to delete upstream of 88 nucleotide position from the first base of the translation initiation codon and make a set of DNAs 10 base pairs shorter than the previous. PCR was performed between the synthetic and the M13 reverse primers (5'-AGCGGATAACAATTTCACACAGGAAAC-3') using pHW257 (5.3kb), which lacked the smaller *Hin*dIII fragment (1.0kb) from pSH253, as a template. The PCR products were cleaved with *Eco*RI, and the *Eco*RI fragments were ligated with the larger fragment (4.4kb) of pSH253

cleaved with *Eco*RI, to produce the deletion plasmids pHW50~57. The correctness of deletions in the plasmids was confirmed by DNA sequencing.

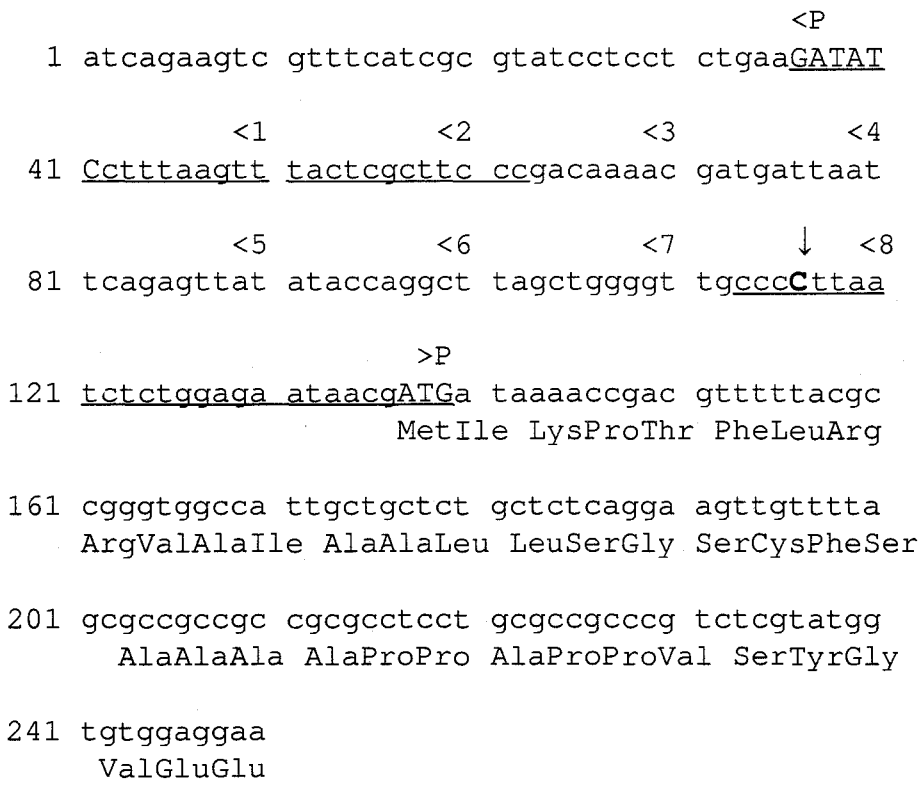

Fig. 1-1. The nucleotide sequence of the 5'-region of *ggt*.
The position of C revealed by the arrow indicates the transcriptional start point. Underlined nucleotides indicate the annealing region with primers for amplifying the *ggt* promoter by PCR. <P P> region indicates the amplified promoter region by PCR for constructing the fusion gene. <1~<8 indicate the annealing sites of 8 synthetic oligonucleotides. The base under the signal < indicates the first 5'-base annealed with oligonucleotides. GATATC and ATG indicate an *Eco*RV site and initiation codon, respectively.

Table 1-2. Synthetic oligonucleotides for the construction of deletion plasmids

No.	Sequences of synthetic oligonucleotides	Resultant plasmids
1	5'-CCGAATTCTTTACTCGCTTCCCGACAAAACGATG-3'	pHW50
2	5'-CCGAATTCTCCCGACAAAACGATGATTAATTCAGAG-3'	pHW51
3	5'-CCGAATTCACGATGATTAATTCAGAGTTATATACCAG-3'	pHW52
4	5'-CCGAATTCATTCAGAGTTATATACCAGGCTTAGCTG-3'	pHW53
5	5'-CCGAATTCATATACCAGGCTTAGCTGGGGTTGC-3'	pHW54
6	5'-CCGAATTCCTTAGCTGGGGTTGCCCCTTAATCT-3'	pHW55
7	5'-CCGAATTCGTTGCCCCTTAATCTCTGGAGAATAAC-3'	pHW56
8	5'-CCGAATTCAATCTCTGGAGAATAACGATGATAAAACC-3'	pHW57

GAATTC; *Eco*RI site.

Construction of plasmids pHW74 and pCG1. To examine effect of promoter exchange on GGT activity, a plasmid pHW74 was constructed as follows. A procaryotic expression vector (pKK223-3) (33), which has *tac* promoter, was cleaved with *Sma*I and *Eco*RI. Plasmid pSH101 (4) was cleaved with *Sma*I and *Eco*RI, and the fragment (2.5kb) containing the gene encoding small subunit was ligated with the *Sma*I-*Eco*RI fragment (4.6kb) of pKK223-3. The resultant plasmid pHW73 (7.1kb) was cleaved with *Eco*RI. Plasmid pHW71 (13, also chapter 2) was cleaved with *Eco*RI, and the smaller *Eco*RI fragment (0.7kb) was ligated with pHW73 cleaved with *Eco*RI. The resultant plasmid pHW74 (7.8kb) had the initiation codon of *ggt* just downstream of *tac* promoter. This was confirmed by DNA sequencing. Plasmid pCG1, which had the entire *ggt* composed of *ggt* promoter and

structural gene, was constructed as follows. Plasmid pSH101 was cleaved with *Sma*I and *Pst*I, and the *Sma*I-*Pst*I fragment (4.4kb) containing the entire *ggt* was ligated with pKK223-3 cleaved with *Sma*I and *Pst*I. The resulting plasmid pCG1 (9.0kb) had the entire *ggt* in the opposite direction from the *tac* promoter. This was confirmed by DNA sequencing.

Construction of plasmid pHW1405. To examine the effect of growth temperature on *ggt* promoter activity, a fusion gene, which contained *lacZ* downstream of the *ggt* promoter was constructed. The prototype plasmid pMC1403 (34) contains the entire *lac* operon but lacks the promoter, operator, and translation initiation sites as well as the first eight nonessential codons of *lacZ* for β-galactosidase. PCR was performed between a primer (5'-CCGAATTCGATATCCTTTAAGTTTACTCGCTTCCC-3') introducing the *Eco*RI site upstream of *ggt* promoter and a primer (5'-CCGGATCCATCGTTATTCTCCAGAGATTAAGGGG-3') introducing the *Bam*HI site downstream of translation initiation site of *ggt*, using pSH253 as a template, to amplify only the *ggt* promoter (Fig. 1-1). The PCR product was cleaved with *Eco*RI and *Bam*HI, and the *Eco*RI-*Bam*HI fragment (0.1kb) was ligated with the larger fragment (9.9kb) of pMC1403 cleaved with *Eco*RI and *Bam*HI, to produce the plasmid pHW1405 (10kb). The insertion of *ggt* promoter in pMC1403 was confirmed by DNA sequencing.

Estimation of mRNA stability of *ggt*. To determine the stability of *ggt*

mRNA at 20℃ and 37℃, a 20-ml culture of strain MG1655 in the exponential phase at 20℃ was treated with 200μg/ml of rifampicin and half of culture was immediately shifted to 37℃. After the addition of rifampicin, 1.0ml aliquots were removed from the cultures at 20℃ and 37℃, respectively, then immediately transferred to liquid N_2. The frozen culture was centrifuged at 11,000 x g at 4℃ for 12min, and the cells were harvested. Total RNA was isolated and 10μg of total RNA was subjected to Northern blot analysis as described above. Hybridized bands were quantified with a BAS2000 Bio Imaging Analyzer (Fuji Photo Film Co.). Graphs of percent of remaining mRNA were plotted against time after addition of rifampicin.

RESULTS

Effect of growth temperature on GGT activity and expression level of GGT protein of some bacteria. *E. coli* K-12 (strain MG1655), *E. coli* B, and *Salmonella typhimurium* were cultured until they reached the stationary phase in LB medium at 20℃ and 37℃, respectively. GGT activity in the periplasmic fraction of each strain was measured (Table 1-3). The GGT activity of each strain grown at 20℃ was higher than that at 37 ℃. The GGT activity of *E. coli* K-12 and *E. coli* B grown at 20℃ was over 6-fold than that at 37℃. Cell free extracts from the same amount of cells of *E.*

Table 1-3. GGT activity of bacterial strains grown at 20 and 37℃

Strain	Temperature (℃)	Specific activity* (mu/mg)
Escherichia coli K-12	20	25.2
	37	2.7
Escherichia coli B	20	1.8
	37	0.3
Salmonella typhimurium	20	25.1
	37	9.7

Strains were cultured in 100ml of LB medium at 20℃ and 37℃. The periplasmic fraction from each strain was obtained by lysozyme treatment, then GGT activity and the protein concentration were measured.
* Data are averages of two independent measurements.
Values agreed ±20%.

coli K-12, *E. coli* B, and *S. typhimurium* grown at 20 and 37℃ were obtained, respectively, and subjected to SDS-PAGE, followed by Western blot analysis using anti-*E. coli* K-12 GGT antibody (Fig. 1-2). The protein in the cell free extracts of *E. coli* B, and *S. typhimurium* that reacted with anti-*E. coli* K-12 GGT antibody had the same mobility as the large and small subunits of *E. coli* K-12 GGT. As shown in Fig. 1-2, *E. coli* K-12, *E. coli* B, and *S. typhimurium* grown at 20℃ produced much more GGT protein than those at 37℃. The ratio of expressed mature GGTs of *E. coli* K-12, *E. coli* B, and *S. typhimurium* grown at 20℃ to those at 37℃ were about 6, 4, and 3, respectively. These values were almost compatible with those of the enzyme activity. There was no remarkable differences among the three strains, in the

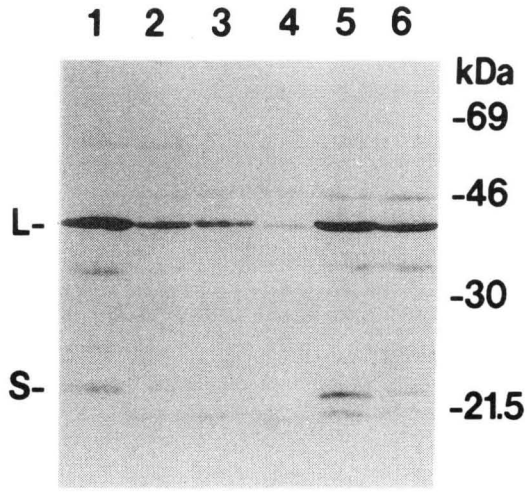

Fig. 1-2. Western blot analysis of cell free extracts from *E. coli* K-12, *E. coli* B, and *S. typhimurium*.
Cell lysates from the same amounts of *E. coli* K-12, *E. coli* B, and *S. typhimurium* grown at 20 and 37℃ were subjected to SDS-PAGE, respectively, followed by Western blot analysis using anti-*E. coli* K-12 GGT antibody. Lane 1, *E. coli* K-12 20℃; lane 2, *E. coli* K-12 37 ℃; lane 3, *E. coli* B 20℃; lane 4, *E. coli* B 37℃; lane 5, *S. typhimurium* 20℃; and lane 6, *S. typhimurium* 37℃.

expression level of precursor GGT which was not processed into large and small subunits. GGT protein in *E. coli* K-12 cells grown at 42℃ was undetectable.

Effect of growth temperature on *ggt* transcripts of *E. coli* K-12.

Total RNA (30μg) was isolated from *E. coli* K-12 (strain MG1655) cells

grown at 20, 37, and 42℃, respectively, and separated by agarose gel electrophoresis, followed by Northern blot analysis. Figure 1-3 shows that cells grown at 20℃ had much more *ggt* transcripts than those at 37℃. No *ggt* transcripts were detected in cells grown at 42℃. The *ggt* mRNA level in the cells was analyzed by densitometry. There was a 10-fold increase in the *ggt* mRNA level of cells grown at 20℃ compared with that at 37℃. This might be caused by an increase in the transcriptional rate or the *ggt* mRNA stability.

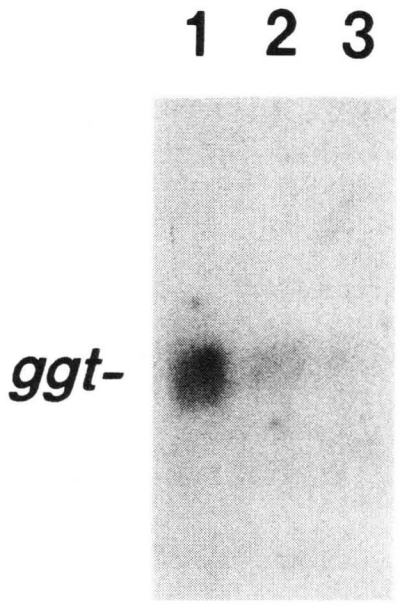

Fig. 1-3. Northern blot analysis of total RNA from *E. coli* K-12 using *ggt* as a DNA probe. Total RNA (30μg) was isolated from *E. coli* K-12 (strain MG1655) cells grown at 20, 37, and 42℃, respectively, and separated by agarose gel electrophoresis, followed by Northern blot analysis using the *ggt* gene as a DNA probe. Lane 1, 20℃; lane 2, 37℃; and lane 3, 42℃.

This abundance of the *ggt* mRNA level at low temperature was compatible with the level of enzymatic activity of GGT.

Identification of 5' end of *ggt* mRNA by primer extension analysis. The 5' end of *ggt* mRNA was mapped by means of primer extension analysis. Only one signal resulted from strain SH642 (Fig. 1-4), and it corresponded to nucleotide C identical to 22nd nucleotide upstream of the first nucleotide A of the initiation codon (Fig. 1-1).

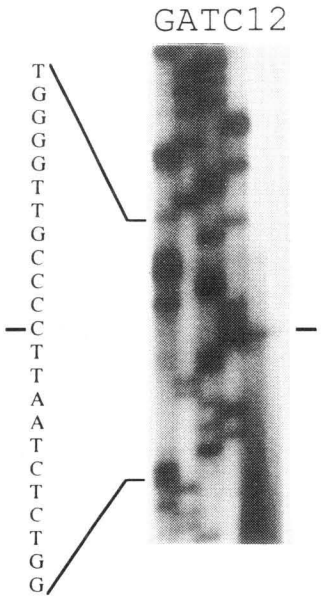

Fig. 1-4. Identification of 5'-end of *ggt* mRNA by primer extension analysis.
Total RNA (50μg) was isolated from *E. coli* K-12 (strain SH642) cells grown at 20 and 37 ℃, respectively, and primer extension analysis was performed as described under Materials and Methods. G, A, T, and C indicate sequence ladder of pSH253, using the extension primer as a sequencing primer. Lane 1, 20℃ and lane 2, 37℃.

The 5' end of the *ggt* mRNA of strain MG1655 was identical to that of strain SH642 (data not shown), and there were no apparent differences in the 5' end of *ggt* mRNA between cells grown at 20℃ and those at 37℃. Upstream of this transcriptional start point, there was no sequence that had high similarity with the consensus promoter sequences.

Identification of promoter region by deletion of 5'-region of *ggt*. A transformant of the GGT-deficient strain SH641 with a plasmid pHW254, which contains the entire *ggt* downstream of the first *Eco*RV site (Fig. 1-1) in pUC119, exhibited higher GGT activity when cultured at 20℃ than at 37℃. Eight mutant plasmids pHW50~57, that contained a deletion set of *ggt* DNAs 10 base pairs shorter than the previous downstream of the first *Eco*RV site, were constructed as described under Materials and Methods. Strain SH641 transformed with plasmids pHW50~57 were named HW360, HW361, HW365, HW366, HW367, HW371, HW372, and HW373, respectively. The GGT activity in the periplasmic fraction from each transformant grown at 20℃ was measured (Fig. 1-5). Strains HW360, 361, and 365 exhibited GGT activity, strains HW366 and 367 exhibited little GGT activity and HW 371, 372, and 373 exhibited none.

Effect of promoter exchange on GGT activity. Strain SH641 was transformed with plasmids pHW74, which had the *tac* promoter upstream of initiation codon of *ggt*, and pCG1, which had original *ggt* promoter upstream

of the initiation codon for *ggt*. Transformants were obtained, and named HW350 and CG3004, respectively. Strains HW350 and CG3004 were cultured in LB medium containing 1mM isopropyl-β-D-thiogalactopyranoside

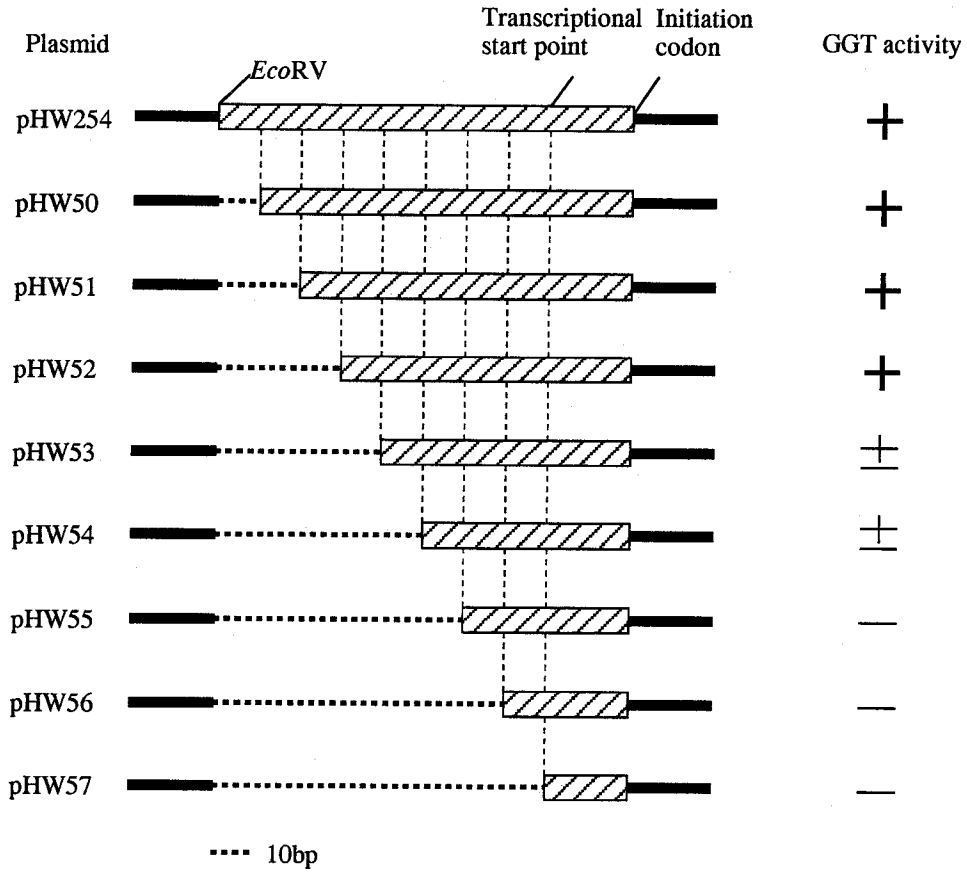

Fig. 1-5. Identification of the promoter region by deleting 5'-region of *ggt*. Eight mutant plasmids, pHW50~57, which contained *ggt* deleted a set of DNAs 10 base pairs sorter than the previous downstream of the *Eco*RV site (Fig. 1-1), were constructed as described under Materials and Methods. Strain SH641 was transformed with these plasmids and grown at 20 ℃. GGT activity was measured in the periplasmic fraction of each transformant. Regions of the shaded box and the dotted line revealed 5'-region of *ggt* and deleted region, respectively.

(IPTG) at 37℃ and the GGT activity in the periplasmic fractions was measured (Table 1-4). As shown in Table 1-4, there was 5-fold GGT activity in strain HW350 grown at 37℃ compared with that in strain CG3004 at 37℃. However, there was little difference in the GGT activity between strains HW350 grown at 20℃ and CG3004 grown at 20℃. Strain HW350 grown at 20℃ had 2.7-fold higher GGT activity than that grown at 37℃.

Table 1-4. Effect of promoter exchange on GGT activity

Strain (promoter)	Temperature (℃)	Specific activity* (mu/mg)
HW350 (*tac* promoter)	20	441
	37	164
CG3004 (*ggt* promoter)	20	313
	37	32

Strains were cultured in 100ml of LB medium containing 1mM IPTG at 20℃ and 37℃. The periplasmic fraction from each was obtained by lysozyme treatment, then GGT activity and protein concentration were measured.

* Data are averages of two independent measurements.
Values agreed ±10%.

β-Galactosidase activity of fusion gene. β-Galactosidase-deficient strain MC1061 *recA* was transformed with pHW1405, which contained *lacZ* downstream of the *ggt* promoter. The transformant was named HW382. Cell free extracts from strain HW382 grown at 20℃ and 37℃ were obtained by

ultrasonic treatment, and the β-galactosidase activity in each was measured (Table 1-5). As shown in Table 1-5, the β-galactosidase activity was 1.6-fold higher in strain HW382 cells grown at 20℃ than in those grown at 37℃. MC1061 *recA* transformed only with the vector plasmid pMC1403 exhibited no β-galactosidase activity. The prototrophic strain MG1655 grown in LB medium containing 1mM IPTG at 37℃ exhibited higher β-galactosidase activity than those at 20℃ (Table 1-5).

Table 1-5. β-Galactosidase activity of strains HW382 and MG1655

Strain (promoter)	Temperature (℃)	Specific activity (mu/mg)
HW382 (*ggt* promoter)	20	0.88
	37	0.53
MG1655 (*lac* promoter)	20	1.62×10^3
	37	2.34×10^3

HW382 was cultured in 100ml of LB medium at 20℃ and 37℃. MG1655 was cultured in 100ml of LB medium containing 1mM IPTG at 20℃ and 37℃. The cell free extract from each was obtained by ultrasonic treatment, then β-galactosidase activity and protein concentration were measured.

Stability of *ggt* transcripts at 20℃ and 37℃. To determine the stability of *ggt* mRNA, RNA synthesis of strain MG1655 was terminated with rifampicin (200μg/ml) and the residual *ggt* mRNA was traced by Northern blot analysis. As shown in Fig. 1-6, the mRNA was much more stable at 20℃

than at 37℃.

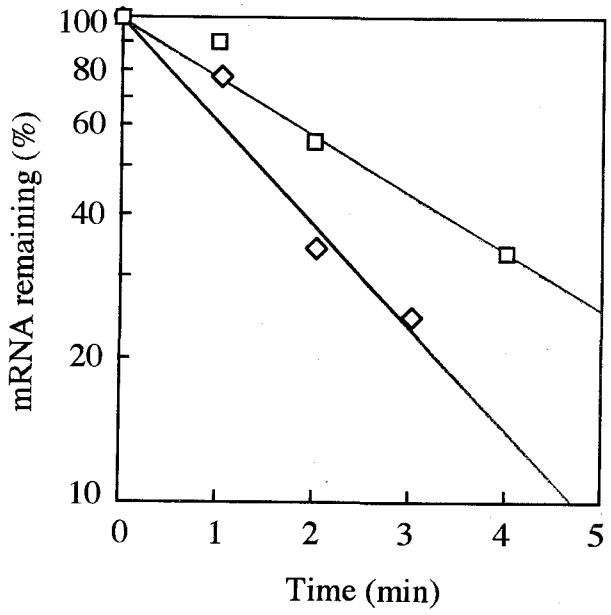

Fig. 1-6. Stability of *E. coli* K-12 *ggt* mRNA.
Northern blot analysis was performed and hybridized bands were quantified. The values were plotted as a percentage of the remaining mRNA versus incubation time after the addition of rifampicin. □ and ◇ indicate the mRNA remaining at 20℃ and 37℃, respectively.

DISCUSSION

E. coli K-12 cells exhibited maximal GGT activity when grown at 20 ℃, whereas at 37℃, the level decreased to 14% of that, although GGT was quite stable at the higher temperature (9). GGT was composed of one large and one small subunit (10), and the nucleotide sequence of its gene revealed

that a signal peptide, as well as the large and small subunits were encoded in a single open reading frame in that order (11). The heterodimer was thought to be synthesized from a single precursor polypeptide via post-translational processing. Therefore, the higher GGT activity in *E. coli* K-12 cells grown at low temperature might be controlled by transcriptional, translational, or processing steps.

Western blot profile (Fig. 1-2) showed that *E. coli* K-12 cells grown at 20℃ had 6-fold more GGT protein than those grown at 37℃. The quantity of precursor GGT in both was very low and there was no significant difference between them. These results indicated that the difference in the GGT activity was due to the expression level of GGT protein, not the processing rate, and that once GGT protein is translated, it is processed immediately. The same phenomena were seen in *E. coli* B and *S. typhimurium*. These three strains may have a common mechanism of GGT expression.

The quantity of *ggt* mRNA in *E. coli* K-12 cells grown at 20℃ was 10-fold more than that grown at 37℃ (Fig. 1-3). Since the difference in GGT protein and activity between *E. coli* K-12 cells grown at 20℃ and 37℃ was almost proportional to that of *ggt* mRNA, the high GGT activity in *E. coli* K-12 cells grown at 20℃ was caused by high *ggt* mRNA levels.

Primer extension analysis and deletion studies of the 5'-region of *ggt* showed that the -35 region of *ggt* promoter region was located between

nucleotides 47 and 37 upstream of the transcriptional start point. The 5' ends of the *ggt* mRNA of strain SH642 grown at 20℃ and 37℃ were identical, indicating that there is only one promoter. However, a typical promoter sequence was not seen in this region. In the neighborhood of the *ggt* promoter region, consensus sequences 5'-CCAAT-3' and 5'-ATTGG-3', to which major cold shock protein CS7.4 is thought to bind (17), were not found. Low temperature-inducible promoters in *E. coli* have been identified, and two were cloned and contained the sequence 5'-CCAAT-3' (18). The expression mechanism of GGT in *E. coli* K-12 is thought to be independent of CS7.4.

Although the *tac* promoter seemed to show reduced efficiency at low temperature (18) and the β-galactosidase activity of prototrophic strain MG1655 grown at 37℃ was higher than that at 20℃ (Table 1-5), strain HW350, which was transformed with the plasmid containing the fusion gene composed of *tac* promoter and GGT structural gene, exhibited higher GGT activity when grown at 20℃ than 37℃ (Table 1-4). Probably this difference in GGT activity according to growth temperature is due to the stability of *ggt* mRNA at low temperature. In fact, the stability of *ggt* mRNA at 20℃ was higher than that at 37℃ (Fig. 1-6).

There are some reports describing another low temperature dependent enzyme, polynucleotide phosphorylase (PNPase) of *E. coli*. Although PNPase was identified as a cold shock protein (14), the expression of PNPase

gene (*pnp*) was not affected by CS7.4 (16), and the stability of *pnp* mRNA was related to the processing at the 5' end by ribonuclease III (RNase III). High level PNPase expression was correlated with the high *pnp* mRNA level because during defective RNase III processing *pnp* mRNA was highly maintained (35). The expression of GGT in *E. coli* might be under similar controls.

The lower growth temperature slightly increased β-galactosidase activity expressed by the fusion gene (Table 1-5), although more native β-galactosidase was expressed in *E. coli* cells grown at 37℃ than that in those at 20℃. Therefore, the higher GGT activity in *E. coli* cells grown at low temperature was due to higher expression levels at low temperature, caused by the higher *ggt* mRNA level resulting from the cold inducible *ggt* promoter. The *ggt* mRNA was also more stable at the lower temperature.

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SUMMARY

Escherichia coli K-12 cultured at 20℃ exhibits higher γ-glutamyltranspeptidase (GGT, EC: 2.3.2.2) activity than that cultured at 37℃ or 42℃. On Western blot analysis, *E. coli* K-12 cells cultured at 20℃ produced more GGT protein than those cultured at 37℃. On Northern blot analysis, mRNA level of GGT gene (*ggt*) in the cells grown at 20℃ was 10-fold higher than that at 37℃. A fusion gene composed of *ggt* promoter and *lacZ* was constructed. There was a little effect of the lower growth temperature on β-galactosidase activity expressed from the fusion gene. The *ggt* mRNA at 20℃ was found to be more stable than at 37℃. These results suggest that the higher GGT activity in *E. coli* cells grown at low temperature was due to higher expression levels at low temperature, caused by the higher *ggt* mRNA level resulting from the cold inducible *ggt* promoter and the stability of *ggt* mRNA at 20℃.

Chapter 2. Effect of Site-Directed Mutations on Processing and Activity of γ-Glutamyltranspeptidase of *Escherichia coli* K-12

γ-Glutamyltranspeptidase (GGT: EC 2.3.2.2), which catalyzes the hydrolysis of γ-glutamyl compounds such as glutathione, and the transfer of their γ-glutamyl moieties to amino acids and peptides, is widely distributed in living organisms and is a key enzyme in glutathione metabolism (1). GGT of *Escherichia coli* K-12 is a periplasmic enzyme, composed of one large subunit and one small subunit (2, 3). The nucleotide sequence of its gene indicates that it codes a signal peptide, large subunit and small subunit in a single open reading frame in this order (4). Therefore, pre-pro-GGT must undergo two proteolytic processing steps to generate its catalytically active dimeric form in the periplasm. At the first step, the signal peptide (25 amino acids) is removed, and at the second step the precursor polypeptide (pro-GGT) is cleaved into a heterodimer. The cleavage site for processing into the large and small subunits is located between the Gln-390 and Thr-391 residues (4). This kind of processing mechanism is very rare in *E. coli,* other examples being penicillin-G acylase (5) and S-adenosylmethionine decarboxylase (6). Previously, during investigation of the catalytic center of GGT, it was found that mutants whose arginyl residues-513 and 571 were substituted with alanyl and glycyl residues, respectively, did not undergo processing and exhibited no

enzymatic activity at all (7, also chapter 3). However, the processing enzyme has not been identified, its localization within the cell has not been determined, and the importance of the amino acid residues at the cleavage site is not understood.

In this chapter, the critical amino acid residues for the processing of *E. coli* GGT and its maturation step were studied by constructing processing-site mutant *E. coli* GGTs by site-directed mutagenesis.

MATERIALS AND METHODS

Materials. Restriction endonucleases, other DNA modification enzymes, sequencing primers and PCR primer were purchased from Takara Shuzo Co., Toyobo Co. and Nippon Gene Co. Anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody (from donkey), and ECL Western blotting detection reagents were purchased from Amersham International plc. A peroxidase immuno-stain set was purchased from Wako Pure Chemical Co. Polyvinylidene difluoride (PVDF) membranes were purchased from Nihon Millipore Kogyo K.K.

Bacterial strains. Strains of *E. coli* K-12 [NS471 (7, also chapter 3): F^- *degP41*(Δ*Pst*I-Kan^r) Δ*lacX74 galE galK thi rpsL* Δ*phoA*(*Pvu*II) *ggt-2 srl300*::Tn*10 recA56*] [SH641 (8): F^- *ggt-2 recA56 rpsL srl300*::Tn*10*] were

used.

Growth medium and cultivation of bacteria. LB broth containing ampicillin at 100 μg/ml was used for preculturing of the strains harboring recombinant plasmids. Overnight precultures were subcultured at a dilution of 1:100 in LB medium and grown at 20℃ for 48h.

Subcloning, transformation and gel electrophoresis. Subcloning, transformation and gel electrophoresis were performed as described (9, 10).

Site-directed mutagenesis. Plasmid pSH253 (11) was used to overexpress wild-type *E. coli* K-12 GGT. The sequences of the mutagenic nucleotides are listed in Table 2-1. *In vitro* mutagenesis of the processing site was performed, using PCR or Oligonucleotide-directed *in vitro* Mutagenesis System Version 2 (Amersham International plc). Mutagenic oligonucleotides were kind gifts from Drs. M. Kobayashi and H. Yukawa (Mitsubishi

Table 2-1. Mutagenic oligonucleotides used in this study.

No.	Sequence of mutagenic oligonucleotides	Amino acid substitution
1	5'-GCAAGCTTGCGCCTTATGAGAGTAATCAAGCTACC-3'	Thr-391→Ala
2	5'-GCAAGCTTGCGCCTTATGAGAGTAATCAATCTACC-3'	Thr-391→Ser
3	5'-GCAAGCTTGCGCCTTATGAGAGTAATCAAACTGCCCAT-3'	Thr-392→Ala
4	5'-GAGAGTAATCAAACTACCGGTTACTCAGTGGTGGATAAA-3'	His-393→Gly
5	5'-GCAAGCTTGCGCCTTATGAGAGTAATGCAACTACC-3'	Gln-390→Ala
6	5'-GCAAGCTTGCGCCTTATGAGAGTAATCAAACTACC CATTACTCAACGGTGGAT-3'	Val-396→Thr

Underlined nucleotides are different from the wild type.

Petrochemical Co.), and H. Matsui (Ajinomoto Co.). PCR was performed between primers 1-3 and 5, and the M13 reverse primer (5'-AGCGGATAACAATTTCACACAGGAAAC-3'), using pSH253 as a template. These mutagenic primers contained a *Hin*dIII site besides the expected mutations. PCR was run for 30 cycles consisting of incubation for 30 seconds at 95℃ for denaturation, 30 seconds at 60℃ for annealing, and 90 seconds at 72℃ for elongation. The PCR products were digested with *Hin*dIII and the smaller *Hin*dIII fragments were ligated with the larger fragment of pSH253 cleaved with *Hin*dIII. The resultant plasmids were cleaved with *Cla*I and the 1.2kb *Cla*I fragments containing the mutations were ligated with the 5.1kb *Cla*I fragment of pSH253. When mutagenic nucleotide 4 was used, oligonucleotide-directed mutagenesis was performed with Oligonucleotide-directed *in vitro* Mutagenesis System Version 2 (Amersham International plc) according to the manufacturer's directions. Nucleotide 4 was designed to generate a new *Cfr*10I site at an expected mutation point for rapid screening of the mutation.

Construction of plasmid pHW71. A procaryotic expression vector (pKK223-3) (12), which has a *tac* promoter, was cleaved with *Sma*I. pSH253 was digested with *Eco*RV and the smaller *Eco*RV fragment (1.2kb) containing N-terminal of *ggt* was ligated with the 4.6kb *Sma*I fragment of pKK223-3. The resultant plasmid (pHW66) (5.8kb) has the initiation codon of *ggt*

downstream of the *tac* promoter. pSH253 was digested with *Hpa*I and *Pst*I, and the smaller *Hpa*I-*Pst*I fragment (1.5kb) was ligated with the larger fragment (5.2kb) of pHW66 cleaved with *Hpa*I and *Pst*I. The resultant plasmid (pHW67) (6.7kb) has the whole structural gene of *ggt* downstream of the *tac* promoter. To construct an *Eco*RI site just upstream of the initiation codon of *ggt*, PCR was performed between a mutagenic primer containing the *Eco*RI site (5'-CTGGAGAATTCCGATGATAAAACCG-3') and the M13 reverse primer, using pSH253 as a template. The PCR product was digested with *Eco*RI and the *Eco*RI fragment (0.7kb) was ligated with the larger fragment (5.9kb) of pHW67 cleaved with *Eco*RI. Finally, the constructed plasmid (pHW71) (6.6kb), which has the initiation codon of *ggt* just downstream of the *tac* promoter, was obtained.

DNA sequence. The correctness of the DNA sequences of the mutant plasmids was confirmed by the dideoxy-chain termination method (13, 14) with a BcaBest Dideoxy Sequencing Kit (Takara Shuzo Co.), using [α-^{32}P]dCTP (ICN Biomedicals Inc.), and an Applied Biosystems (Division of Perkin Elmer Co.) Model 373A automated DNA sequencer according to the recommended protocols.

Cell fractionation. Cell fractionation was performed as described, with slight modifications (2). The *E. coli* K-12 cells were grown in 100ml of LB medium, harvested, and then washed with distilled water. The harvested cells

were suspended in 3.5ml of distilled water. Two ml of 0.1M Tris-HCl (pH8.3), 1.8ml of 2M sucrose, 0.35ml of 1% EDTA (pH7.0), and 0.35ml of 0.5% lysozyme in 10mM Tris-HCl (pH8.3), in that order, were added to the suspension, and incubation was conducted at 30℃ for 70 min. The suspension was centrifuged at 18,000 x g for 20 min. The supernatant (periplasmic fraction) was dialyzed against 20mM Tris-HCl (pH8.3). The precipitate (spheroplasts) was disrupted at 4℃ in 8ml of 5mM $MgCl_2$, twice. The burst spheroplast suspension was centrifuged at 18,000 x g for 20 min. The supernatant (cytoplasmic fraction) was obtained. The precipitate (membrane fraction) was suspended in 2ml of 1% EDTA (pH7.0) and then homogenized with a Teflon homogenizer.

Protein determination. The protein concentration was determined by the method of Lowry *et al.* (15), with bovine serum albumin as a standard. Insoluble protein was determined by a modified method. A 0.01-ml portion of 10N NaOH was added to 0.09ml of a sample, and the mixture was left to stand for 30 min at room temperature for solubilization. Next, 0.98ml of 2% Na_2CO_3 and 0.02ml of Folin B reagent were added to the mixture, followed by incubation at 37℃ for 10 min. A 0.1-ml aliquot of phenol reagent was added to the mixture, followed by incubation at 37℃ for 20 min. The absorbance at 610nm was measured.

Electrophoresis and GGT activity staining. Sodium dodecyl sulfate-

polyacrylamide gel electrophoresis (SDS-PAGE) and native polyacrylamide gel electrophoresis (native PAGE) were performed as described previously (16, 17). Staining for GGT activity on native polyacrylamide gel was performed as described previously (18).

Western blotting. Each cell fraction was subjected to SDS-PAGE and native PAGE, and then electroblotted onto a PVDF membrane (19). After the PVDF membranes had been blocked with TBS-T (Tris-buffered saline, pH7.6, and 0.1% Tween 20) containing 5% skim milk, for 60 min at room temperature, they were incubated with a rabbit anti-*E. coli* GGT antibody for 60 min at room temperature. After the membranes had been washed with TBS-T, they were incubated with anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody (from donkey), for 20 min at room temperature. The membranes were washed with TBS-T, and *E. coli* GGT was stained with the peroxidase immuno-stain set or detected with ECL Western blotting detection reagents, then the membranes were washed with water and air-dried.

GGT activity. GGT activity was measured as described (2). The activity was determined with L-γ-glutamyl-*p*-nitroanilide (γ-Glu-*p*NA) as a substrate and glycylglycine (Glygly) as an acceptor. The assay solution contained 0.25μmol of γ-Glu-*p*NA, 30μmol of Glygly, 25μmol of Tris-HCl (pH8.73) and enzyme in a final volume of 0.5ml. After incubation at 37℃ for 15 min,

the reaction was terminated by addition of 1ml of 3.5N acetic acid and the absorbance at 410nm was measured.

Determination of specific activity and processing efficiency on Western blot analysis. The Western blot profiles were read with a scanner, and the densities of these protein bands were quantified with a Macintosh Centris 650 computer using the NIH Image 1.54 Program. Specific activity was calculated as activity divided by density of mature GGT. Processing efficiency was calculated as density of mature GGT divided by density of the total GGT.

RESULTS AND DISCUSSION

GGT of *E. coli* K-12 is thought to be synthesized as a single polypeptide and then processed into heterodimeric form. In order to investigate the maturation step, the authors sought the precursor in various cell fractions of these strains. Wild-type *E. coli* K-12 and a GGT-overexpressing transformant (strain SH642) produce much more GGT in culture at 20℃ than at 37℃ (2, 8). However, the precursor could scarcely be detected in any fraction of the cells of either strain cultured at 20℃ (data not shown). In order to produce much more GGT at 37℃, plasmid pHW71 which has a *tac* promoter upstream of the GGT structural gene instead of the original *ggt*

promoter, was constructed as described under Materials and Methods. A GGT-deficient mutant (strain SH641) was transformed with pHW71 and the transformant was named strain HW344. Strain HW344 had higher GGT activity at 37℃ than the transformant of SH641 with pSH253. Strain HW344 was grown at 37℃ in LB broth containing isopropyl-β-D-thiogalactopyranoside at 1mM, and the cells were harvested at the exponential phase. The cells were fractionated into periplasmic, membrane and cytoplasmic fractions, and then these fractions were subjected to SDS-PAGE, followed by Western blot analysis (Fig. 2-1). The periplasmic fraction only contained the mature GGT. The precursor of GGT was found in the membrane fraction, and no precursor of GGT was detected in the cytoplasmic fraction. The membrane fraction contained the precursor only, and these results clearly indicate the existence of a proteolytic processing step for the maturation of GGT. Taking into consideration that the mutation at the arginyl residue-571 near the C-terminal, which was substituted with a glycinyl residue, prevented maturation of GGT (7, also chapter 3), GGT may be folded into the appropriate conformation after its precursor has passed through the membrane, and then it may be processed into the large and small subunits.

Sequence alignment, at the processing site, of GGTs is shown in Table 2-2. The N-terminal regions of the small subunit of GGTs, on the right side of the arrow, exhibit high similarity, though the C-terminal regions of the

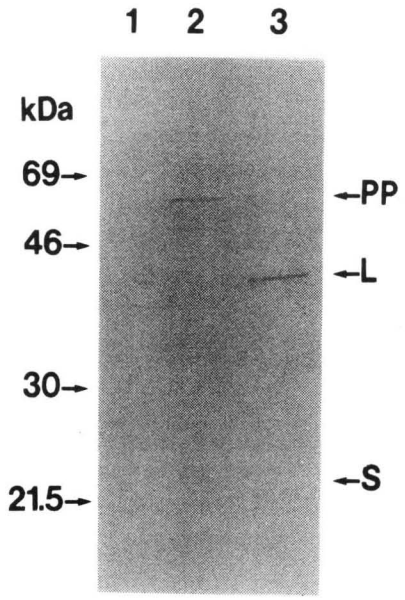

Fig. 2-1. Western blot analysis of cell fractions of strain HW344.
Cell fractionation of strain HW344 was performed, and each fraction was subjected to SDS-PAGE, followed by Western blot analysis. Lane 1, cytoplasmic fraction (5μg of protein); 2, membrane fraction (55.6μg of protein); 3, periplasmic fraction (5μg of protein). L, S, and PP indicate the positions of the large subunit, small subunit, and pre-pro-GGT, respectively.

large subunit, on the left side of the arrow, are less conserved. In particular, the N-terminal amino acid residues of the small subunits of all GGTs are threonyl ones. Therefore, in order to determine the critical amino acid residues for GGT processing, *in vitro* mutagenesis at threonyl residue-391, threonyl residue-392, histidinyl residue-393, glutaminyl residue-390, and valinyl residue-396 was performed.

Table 2-2. Sequence alignment at the processing site among GGTs.

Origin	Sequence at the processing site	Reference
	390 ↓ 391*1	
E. coli GGT	ESNQ TTHYSVVDK	(4)
Pseudomonas A14 GGT	EGSN TTHYSIVDK	(20)
B. subtilis GGT	QEGQ TTHFTVTDR	(21)
B. natto NR-1 GGT*2	TTHFAVTWR	(22)
rat GGT	DDGG TAHLSVVSE	(23)
human GGT	DDGG TAHLSVVAE	(24)
porcine GGT	DDAG TAHLSVVSD	(25)
human GGT-related enzyme	HGTG TSHVSVLGE	(26)

*1 Amino acid residue number of *E. coli* GGT.

*2 Determined by a protein sequencer.

Mutant plasmids with a mutation at the processing site were constructed, as described under Materials and Methods. The mutations were confirmed by determination of DNA sequence; only the mutation points were different from the wild-type plasmid. In these experiments, pSH253 composed of the native *ggt* containing the original promoter was employed as the wild-type plasmid, since it did not produce the precursor. The mutant plasmid whose threonyl residue-391 was substituted with an alanyl residue was designated as pT391A. In the same way, each constructed plasmid was named on the basis of the substituted amino acid. DegP protease-deficient strain NS471, which stabilized ordinarily unstable cell envelope proteins such

as the precursor, was transformed with each plasmid and the transformant was named based on the amino acid substitution. For example, the strain harboring pT391A was named T391A.

The periplasmic fraction was obtained from each transformant and a total of 20μg of protein of each periplasmic fraction was subjected to SDS-PAGE, followed by Western blot analysis (Fig. 2-2). The wild-type strain harboring pSH253 gave only two bands corresponding to the large and small subunits, though the latter band was not clear since its antigen activity was

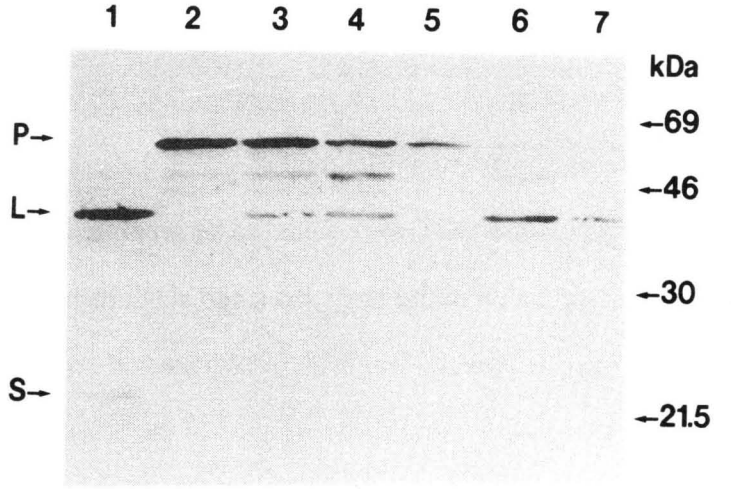

Fig. 2-2. Western blot analysis of periplasmic fractions of mutants as to processing site I. Periplasmic fractions of mutants (20μg of protein) were subjected to SDS-PAGE, followed by Western blot analysis. Lane 1, wild-type strain; 2, strain T391A; 3, strain T391S; 4, strain T392A; 5, strain H393G; 6, strain Q390A; 7, strain V396T. L, S, and P indicate the positions of the large subunit, small subunit, and pro-GGT, respectively.

always low. Strains T391A and H393G gave only a single band corresponding to a molecular weight of 59kDa. Since mature GGT consists of one large (39kDa) and one small (20kDa) subunit, strains T391A and H393G only synthesized precursor GGT, which was not processed into the large and small subunits. Strains T391S and T392A produced more precursor and less mature GGT. Strain Q390A produced less precursor and more mature GGT. Strain V396T only produced mature GGT, like the wild type, though valinyl residue-396 is a highly conserved residue among GGTs. Strains T391A, T391S, T392A, and Q390A gave a band corresponding to a molecular weight of 48kDa. This protein might be derived from the precursor by abnormal proteolysis because of the site-directed mutations.

A total of 20μg of protein of each periplasmic fraction was subjected to native PAGE, followed by Western blot analysis (Fig. 2-3). As shown in Fig. 2-3, precursor and mature GGT were found to be separated. The molecular weight determination of the large and small subunits using ion-spray mass spectrometry suggested that no fragment of peptide was removed by the processing (chapter 4). Since the molecular weights of the precursor and mature GGT should be identical, the difference in mobility suggested that the folding and/or electric charge on the molecular surface were different. The effect of the mutations on the processing shown in Fig. 2-3 is consistent with the results in Fig. 2-2.

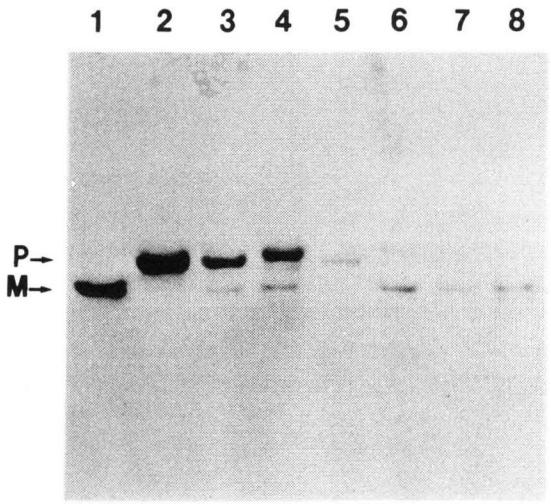

Fig. 2-3. Western blot analysis of periplasmic fractions of mutants as to processing site II. Periplasmic fractions of mutants (20μg of protein) were subjected to native PAGE, followed by Western blot analysis. Lane 1, wild-type strain; 2, strain T391A; 3, strain T391S; 4, strain T392A; 5, strain H393G; 6, strain Q390A; 7, strain V396T; 8, purified native GGT (60ng). P and M indicate the positions of pro-GGT and mature GGT, respectively.

The GGT activity of the periplasmic fractions of the mutants was measured and is shown as a percentage of the wild-type value (Table 2-3). Strains T391A and H393G which produced only the precursor and no mature GGT exhibited no GGT activity. Strains T391S and T392A, which produced only a little mature GGT exhibited a little GGT activity. Strains Q390A and V396T, which produced a larger amount of mature GGT exhibited GGT activity. A comparison of GGT activity and processing efficiency among

Table 2-3. Comparison of GGT activity and processing efficiency among mutants.

Strain	GGT activity (%)	Processing efficiency (%)
Wild type	100	100
T391A	0	0
T391S	11.5	11.8
T392A	10.4	16.8
H393G	0	0
Q390A	38.6	81.9
V396T	53.7	100

GGT activity of periplasmic fraction of each strain was measured with γ-Glu-*p*NA as a substrate and Glygly as an acceptor, and the processing efficiency was determined from the Western blot profiles (Fig. 2-3).

mutants is shown in Table 2-3. The processing efficiency of the mutants was calculated from the Western blot profiles in Fig. 2-3.

Since T391A and H393G mutations completely prevented the processing, and the threonyl-391 and histidinyl-393 residues are conserved in all GGTs whose sequences are known, these two residues are critical for GGT processing. Since T392A mutation almost entirely prevented processing, threonyl residue-392 is also important. As judged from a comparison of GGT activity and processing efficiency, GGT activity is roughly proportional to the processing efficiency, and it is strongly suggested that the precursor is an inactive form.

A total of 100μg of protein of each periplasmic fraction was subjected

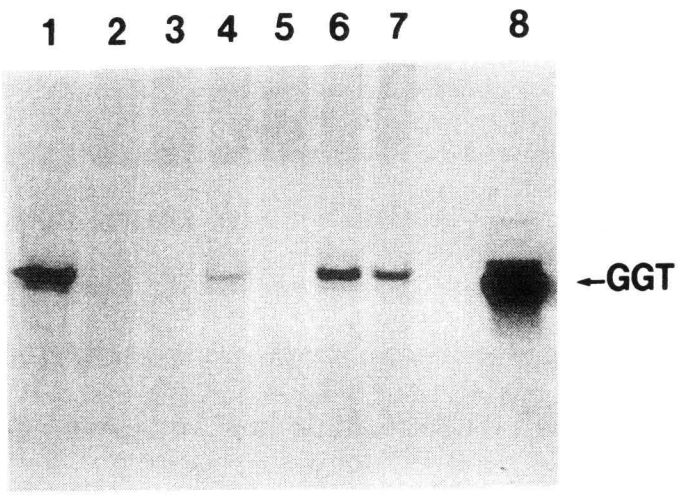

Fig. 2-4. Staining of native polyacrylamide gel for GGT activity.
Periplasmic fractions of mutants (100μg of protein) were subjected to native PAGE, followed by staining for GGT activity. Lane 1, wild-type strain; 2, strain T391A; 3, strain T391S; 4, strain T392A; 5, strain H393G; 6, strain Q390A; 7, strain V396T; 8, purified native GGT (50μg). GGT indicates the position of the native GGT.

to native PAGE, followed by staining for GGT activity (Fig. 2-4). The wild-type strain and strains T392A, Q390A and V396T, all of which produced mature GGT, exhibited a red band of activity with the same mobility as purified native GGT. Because the amount of mature GGT of strain T391S was small, strain T391S did not show the band. Precursor from strains T391A, T391S, T392A, H393G, and Q390A showed no activity band. It has been reported that HepG2 GGT possesses GGT activity, though it was constructed as a

single chain which was not processed into heterodimeric form (27, 28). Mutant precursors of *E. coli* K-12 GGT exhibited no enzymatic activity. Strains T392A and Q390A produced both precursor and mature GGT, and only the mature GGT exhibited enzymatic activity. These results indicate that the reason why the precursor exhibited no enzymatic activity might be inhibition of processing into heterodimeric form, rather than an effect of the site-directed mutation. Processing into heterodimeric form and formation of the active center seem to be essential to expression of the GGT activity of *E. coli* K-12.

The C-terminal amino acid residue of the large subunit, glutaminyl residue-390, is not a conserved residue, and strain Q390A showed a similar phenotype to the wild type. This suggests that the C-terminal side of the large subunit is not so important for the processing as the N-terminal side of the small subunit.

When human hepatoma HepG2 GGT cDNA and rat renal GGT cDNA were expressed in *E. coli* cells, the expressed products were a single polypeptide chain, identified as the precursor, and two polypeptides, identified as the precursor and a GGT large subunit-like peptide, respectively (29, 30). The reason why mammalian GGT cDNA is difficult to express as the mature form in *E. coli* cells might be that mammalian GGTs have an alanyl residue in place of threonyl residue-392 in *E. coli* GGT. This is compatible with the

conclusion in this chapter that the sequence of threonyl-threonyl-histidinyl residues at the N-terminal of the small subunit is very important for the processing of *E. coli* GGT.

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SUMMARY

γ-Glutamyltranspeptidase (GGT; EC 2.3.2.2) of *Escherichia coli* K-12 is thought to be synthesized from a single precursor polypeptide into a heterodimeric form through post-translational processing. Amino acid residues at the cleavage site for processing into the large and small subunits were substituted by site-directed mutagenesis. The processing phenotypes of six mutants were examined by Western blot analysis, and their GGT activities were measured. Mutations at the N-terminal amino acid residues of the small subunit (Thr-391, Thr-392, and His-393) prevented the maturation of the enzyme and the immature mutants exhibited no enzymatic activity. A mutation at the C-terminal residue of the large subunit (Gln-390) had less effect on the processing and enzymatic activity. These results suggest that the sequence of threonyl-threonyl-histidinyl residues at the N-terminal of the small subunit is very important for the processing of *E. coli* K-12 GGT and this processing is essential to the expression of GGT activity of *E. coli* K-12.

Chapter 3. Site-Directed Mutations at Arg-114, 513, and 571 of γ-Glutamyltranspeptidase of *Escherichia coli* K-12

γ-Glutamyltranspeptidase (GGT: EC 2.3.2.2), which catalyzes the hydrolysis of γ-glutamyl compounds such as glutathione, and the transfer of their γ-glutamyl moieties to amino acids and peptides, is widely distributed in living organisms and has been designated as the key enzyme in glutathione metabolism (1). GGT of *Escherichia coli* K-12 plays a role in utilization of exogenous γ-glutamyl peptides as an amino acid source (2), is localized in the periplasm (3), and is composed of one large subunit and one small subunit (4). The nucleotide sequence of its gene has been reported, and a signal peptide, large subunit, and small subunit have been shown to be encoded in a single open reading frame in this order (5).

Three subsites have been identified in the active center of GGT (1), one was the γ-glutamyl donor site and the others were acceptor sites. Treatments of mammalian GGTs with chemical modifying agents for arginyl residues were reported to inactivate them (6, 7, 8), and those were proposed that an arginyl residue in the small subunit is involved in the recognition of an anionic moiety of an acceptor in the transfer reaction by way of an electrostatic interaction, and that arginyl residue-111 of rat GGT interacts glutathione. Arginyl residue-107 of human GGT was reported to play a significant role in

binding substrate, by site-directed mutagenesis (9).

The amino acid sequence of *E. coli* GGT shows 33.0% homology with those of rat GGT (10) and 32.4% with human GGT (11), and the other amino acid residues are almost all conserved through the conservative substitutions of amino acids. On amino acid sequence alignment for examination of the similarity among the *E. coli*, rat and human GGTs, it was found that arginyl residue-114 of *E. coli* GGT corresponds to arginyl residue-107 of human GGT and two arginyl residues-513 and 571, located on the small subunit are conserved in all of them.

In this chapter, mutant *E. coli* GGTs with arginyl residues-114, 513, and 571 substituted with another amino acid residue by site-directed mutagenesis. The mutant GGTs were compared with the wild-type GGT.

MATERIALS AND METHODS

Materials. Restriction endonucleases, other DNA modification enzymes, sequencing primers and PCR primer were purchased from Takara Shuzo Co., Toyobo Co., and Nippon Gene Co. Anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody (from donkey) was purchased from Amersham International plc. A peroxidase immuno-stain set was purchased from Wako Pure Chemical Co. Polyvinylidene difluoride (PVDF) membranes

were purchased from Nihon Millipore Kogyo K.K.

Bacterial strains. *E. coli* K-12 strains used in this chapter are listed in Table 3-1.

Table 3-1. *E. coli* strains used.

Strain	Genotype	Source or reference
SH641	F⁻ *ggt-2 recA56 rpsL srl300*::Tn*10*	(12)
MV1184	△(*srl-recA*)306::Tn10 △(*lac-proAB*) *ara thi rpsL* ϕ80d*lacZ*△M*15* [F' *proAB lacI*^{q} *lacZ*△M*15 traD*36]	Takara Shuzo Co.
KS474	F⁻ *degP41*(△*Pst*I-Kan^r) △*lacX74* *galE galK thi rpsL* △*phoA*(*Pvu*II)	(13)
SH682	F⁻ *ggt-2 zhg*::Tn*10*	(14)
NS453	F⁻ *degP41*(△*Pst*I-Kan^r) △*lacX74* *galE galK thi rpsL* △*phoA*(*Pvu*II) *ggt-2 zhg*::Tn*10*	This chapter
JC10240	HfrPO45 *srl300*::Tn*10 recA56* *thr300 ilv318 rpsE300*	(15)
NS471	F⁻ *degP41*(△*Pst*I-Kan^r) △*lacX74* *galE galK thi rpsL* △*phoA*(*Pvu*II) *ggt-2 zhg*::Tn*10 recA56*	This chapter

Growth medium and cultivation of bacteria. LB broth containing ampicillin at 100μg/ml was used for preculturing of the strains harboring recombinant plasmids. Overnight precultures were subcultured at a dilution of 1:100 in LB medium and grown at 20℃ two overnights.

Strain construction. The P1 *vir* phage was grown on *E. coli* strain

SH682. The lysate was used to transduce KS474 to obtain Ggt$^-$ Tet^r transductant NS453. A spontaneous tetracycline-sensitive derivative of the transductant was selected according to Bochner *et al.* (16) and rendered *recA* with JC10240, as described (15). The *recA* exconjugant was screened on an LB plate containing 2μg/ml nitrofurantoin (17).

Subcloning, transformation, and gel electrophoresis. Subcloning, transformation, and gel electrophoresis were performed as described (18, 19).

Polymerase chain reaction (PCR). PCR reaction mixtures contained 10~100ng of template DNA, 20pmol of primers and 2units of *Taq* DNA polymerase in 100μl of PCR buffer consisting of 20mM Tris-HCl (pH8.3), 1.5mM $MgCl_2$, 25mM KCl, 100μg/ml bovine serum albumin and 50μM dNTP. Each of the total of 30 cycles of PCR consisted of incubation for 30 sec at 95℃ for denaturation, 30 sec at 60℃ for annealing, and 90 sec at 72℃ for elongation.

Site-directed mutagenesis. Plasmid pSH253 (20) was used to overexpress wild-type *E. coli* K-12 GGT. The sequences of the mutagenic nucleotides are listed in Table 3-2. *In vitro* mutagenesis of arginyl residue-114 was performed using PCR. Mutagenic oligonucleotides were kind gifts from Drs. M. Kobayashi and H. Yukawa (Mitsubishi Petrochemical Co.). PCR was performed between primers 1-4, and the M13 reverse primer (5'-AGCGGATAACAATTTCACACAGGAAAC-3'), using pSH253 as a

template. These mutagenic primers contained a *Cla*I site besides the expected mutations. The PCR products were digested with *Cla*I and the larger *Cla*I fragments (1.2kb) were ligated with the larger fragment (5.1kb) of pSH253 cleaved with *Cla*I. The resultant plasmids were cleaved with *Hin*cII and the 1.5kb *Hin*cII fragments were ligated with the 4.8kb *Hin*cII fragment of pSH253. In the case of arginyl residues-513 and 571, oligonucleotide-directed mutagenesis was performed with a T7-GEN *In Vitro* Mutagenesis Kit (United States Biochemical Co.) and with an Oligonucleotide-directed *in vitro* Mutagenesis System Version 2 (Amersham International plc) according to the manufacturers' directions, using mutagenic oligonucleotide primers 5 and 6 (Table 3-2).

Table 3-2. Mutagenic oligonucleotides used in this study.

No.	Sequences of mutagenic oligonucleotides	Amino acid substitutions
1	5'-CTATCGATTTCC**A**CGAAATGGCAC-3'	Arg-114→His
2	5'-CTATCGATTTCC**A G**GAAATGGCAC-3'	Arg-114→Gln
3	5'-CTATCGATTTC**AAA**GAAATGGCACCC-3'	Arg-114→Lys
4	5'-CTATCGATTTC**GAA**GAAATGGCACCC-3'	Arg-114→Glu
5	5'-ACCAATGCGCCG**G C**TTTCCAC-3'	Arg-513→Ala
6	5'-ATCCGACCCG**G**GCTCGGTGGA-3'	Arg-571→Gly

Bold nucleotides are different from the wild type.
GCCG**G C**, recognition site of *Nae*I; CCCG**G**G, recognition site of *Sma*I.

DNA sequence. The correctness of the DNA sequences of the mutant

plasmids was confirmed by the dideoxy-chain termination method (21, 22) with a BcaBest Dideoxy Sequencing Kit (Takara Shuzo Co.), using [α-^{32}P]dCTP (ICN Biomedicals Inc.).

Preparation of periplasmic fraction. Periplasmic fraction was obtained by lysozyme treatment, as described previously (3).

Purification of GGT. GGT was purified from the periplasmic fraction to electrophoretic homogeneity by lysozyme treatment, ammonium sulfate precipitation, and chromatofocusing (12).

Protein concentration and GGT activity determination. The protein concentration was determined by the method of Lowry *et al.* (23) with bovine serum albumin as a standard. GGT activity was measured as described previously (3). The activity was determined with L-γ-glutamyl-*p*-nitroanilide (γ-Glu-*p*NA) as a substrate and glycylglycine (Glygly) or phenylalanine (Phe) as an acceptor. The assay solution contained 0.25μmol of γ-Glu-*p*NA, 30μmol of Glygly or 10μmol of Phe, 25μmol of Tris-HCl (pH8.73) and enzyme in a final volume 0.5ml. After incubation at 37℃, the reaction was terminated by addition of 1ml of 3.5N acetic acid or citrate buffer (pH2.2) for amino acid analysis solution (Nacalai Tesque, Inc.) and the absorbance at 410nm was measured.

Electrophoresis and Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described

previously (24). Periplasmic fraction from each strain was subjected to SDS-PAGE, and then electroblotted onto a PVDF membrane (25). After the PVDF membranes had been blocked with TBS-T (Tris-buffered saline, pH7.6, and 0.1% Tween 20) containing 5% skim milk, for 60 min at room temperature, they were incubated with a rabbit-anti *E. coli* GGT antibody for 60 min at room temperature. After the membranes had been washed with TBS-T, they were incubated with anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody (from donkey), for 20 min at room temperature. After the membranes had been washed with TBS-T, *E. coli* GGT was stained with the peroxidase immuno-stain set, and then the membranes were washed with water and air-dried.

Amino acid analysis. Enzymatic reaction solutions were analyzed with amino acid analyzer (Shimadzu Co., SCL-6B) equipped with a cation exchange column of Amino-NA (Shimadzu Co.).

RESULTS AND DISCUSSION

Site-directed mutation at arginyl residue-114. Arginyl residue-107 of human GGT was reported to play a significant role in binding substrates (9). Sequence alignment among some GGTs at arginyl residue-114 site of *E. coli* GGT, which corresponds to arginyl residue-107 of human GGT, is shown

in Table 3-3. There is a high similarity in this region among GGTs. So, in order to study whether arginyl residue-114 of *E. coli* GGT has a relation to bind substrates, arginyl residue-114 was substituted with glutamyl lysil, glutaminyl, and histidinyl residues, respectively, by site-directed mutagenesis using PCR.

Table 3-3. Sequence alignment at Arg-114 site among GGTs.

Origins	Sequences at Arg-114 site	References
	↓ 114 *1	
E. coli GGT	IDFREMAPAKATRDMF	(5)
Pseudomonas A14 GGT	LDFREKAPLAATANMY	(26)
B. subtilis GGT	IDSRERAPAGATPDMF	(27)
rat GGT	INAREMAPRLANTSMF	(10)
human GGT	INAREVAPRLAFATMF	(11)
porcine GGT	INAREVAPRLASASMF	(28)
human GGT-related enzyme	INARETVPASHAPSLL	(29)

*1 Amino acid residue number of *E. coli* GGT

Mutant plasmids with a mutation at arginyl residue-114 were constructed, as described under Materials and Methods. The mutations were confirmed by determination of DNA sequence in which only the mutation points are different from the wild-type plasmid. The mutant plasmid whose arginyl residue-114 was substituted with a glutamyl residue was designated as pR114E. In the same way, each constructed plasmid was named on the basis

of the substituted amino acid. GGT deficient strain SH641 was transformed with each plasmid and the transformant was named based on the amino acid substitution. For example, the strain harboring pR114E was named R114E.

The periplasmic fraction was obtained from each transformant and a total 20μg of protein of each periplasmic fraction was subjected to SDS-PAGE, followed by Western blot analysis (Fig. 3-1). All mutant strains gave the same two bands corresponding to the large and small subunits as the wild-type strain harboring pSH253, though the latter band was not clear since its antigen activity was always low.

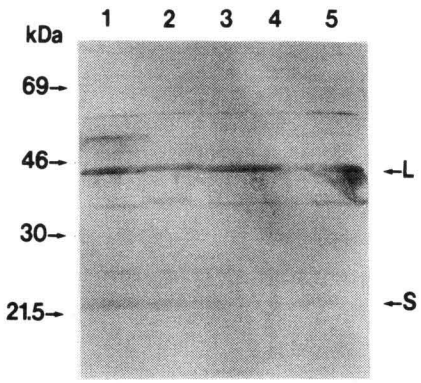

Fig. 3-1. Western blot analysis of periplasmic fractions of mutants as to arginyl residue-114 site.
Periplasmic fractions of mutants (20μg of protein) were subjected to SDS-PAGE, followed by Western blot analysis. Lane 1, wild-type strain; 2, strain R114E; 3, strain R114K; 4, strain R114Q; 5, strain R114H. L and S indicate the positions of the large subunit and small subunit, respectively.

The GGT activity of the periplasmic fractions of the mutants was measured. All mutants exhibited γ-glutamyl hydrolysis activity, however, there was smaller difference of γ-glutamyl hydrolysis activity of them between the presence and the absence of an acceptor in enzymatic reaction solution than that of wild-type strain. Especially, in the case of strain R114Q, no difference was observed (Table 3-4). This result suggested that the mutant of R114Q strain exhibited no γ-glutamyltranspeptidase activity though it possessed γ-glutamyl hydrolysis activity. In order to investigate either of the absence of transpeptidase products or the existence, enzymatic reaction solutions were performed by amino acid analyses.

Table 3-4. Comparison of GGT activity of periplasmic fraction among mutants.

Strains	Specific activity (mu/mg)		a*1/b*2
	GGT	Hydrolysis	
Wild type	143	35.5	5.02
R114H	47.5	24.2	2.97
R114Q	0.97	10.7	1.09
R114K	120	35.2	4.41
R114E	24.3	9.79	3.48

* 1: a, the amount of released *p*NA in the presence of Glygly.

* 2: b, the amount of released *p*NA in the absence of Glygly.

At first, mutant GGT of strain R114Q was purified by lysozyme treatment, ammonium sulfate precipitation, and chromatofocusing. Purified mutant GGT from strain R114Q also showed no difference of γ-glutamyl hydrolysis activity between the absence and the presence of glycylglycine and phenylalanine, respectively, as an acceptor. Enzymatic reaction solutions of purified mutant and native GGTs were examined by amino acid analyses. Analyses of phenylalanine used as the acceptor were shown in Fig. 3-2. Contrary to expectation, the mutant GGT produced transferred compound, that is, γ-glutamyl-phenylalanine. However, the ratio of transpeptidation of the mutant GGT, which was calculated as the amount of γ-glutamyl-phenylalanine

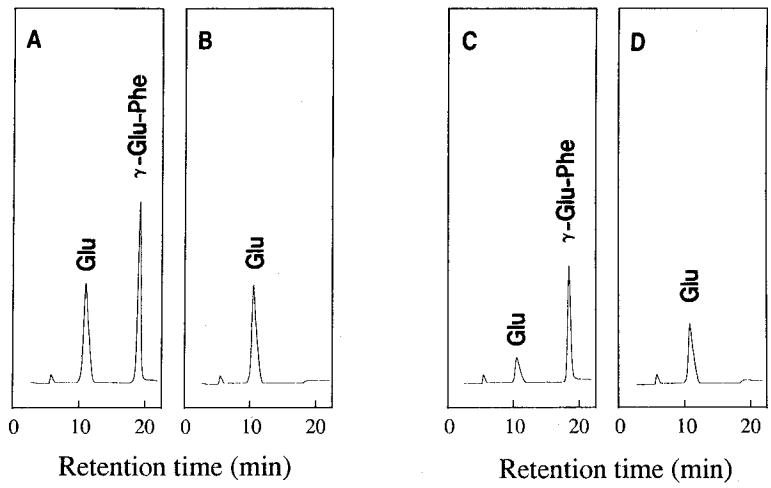

Fig. 3-2 Amino acid analyses of the reaction mixture with mutant R114Q and native GGT. The raction and analyses were carried out as described under Materials and Methods. A, native GGT with an acceptor; B, native GGT without an acceptor; C, mutant GGT of R114Q with an acceptor; D, mutant GGT of R114Q strain without an acceptor.

formed with phenylalanine as an acceptor divided by the amount of hydrolyzed substrate without the acceptor, was 0.45. This value was about half of that of the wild-type GGT of 0.8.

These results suggest that arginyl residue-114 of *E. coli* GGT was not so important for catalyzing hydrolysis of γ-glutamyl linkage, and that the mutant GGT of strain R114Q may have more difficulty in binding an acceptor or the mutant GGT binding an acceptor may have more difficulty in binding a substrate than the wild-type GGT.

Site-directed mutation at arginyl residues-513 and 571. The MV1184 strain harboring pSH253 was infected with the M13KO7 helper phage and single-stranded pSH253 was purified from the phage particles. This ssDNA was used as the mold and the oligonucleotides shown in Table 3-2 were used as primers. Mutant plasmids were constructed by oligonucleotide-directed *in vitro* mutagenesis. Since the oligonucleotides were designed so as to make new restriction endonucleases cleavage sites for *Nae*I and *Sma*I when arginyl residue-513 and arginyl residue-571 were substituted with an alanyl residue and a glycyl residue, respectively, the mutations were confirmed by the restriction pattern of the plasmids on agarose gel electrophoresis. We also confirmed the mutations by DNA sequencing. The mutant plasmid whose arginyl residue-513 was substituted with an alanyl residue was designated as pR513A and that whose arginyl residue-571 was substituted with a glycyl

residue as pR571G.

DegP protease deficient strain NS471 was transformed with pSH253, pR513A, and pR571G. Periplasmic fractions were obtained from each transformants and their GGT activity was measured. The strains harboring pR513A and pR571G showed no GGT activity, which is compatible with the results of a chemical modification study.

The periplasmic fractions were subjected to SDS-PAGE, followed by Western blot analysis (Fig. 3-3). The strain harboring pSH253 gave bands corresponding to the large and small subunits. However, the strains harboring pR513A and pR571G only gave a single band corresponding to molecular weight of 59kDa. A similar results were obtained when whole cells were subjected to SDS-PAGE, followed by Western blot analysis (data not shown).

Since mature GGT consists of one large (39kDa) and one small (20kDa) subunit (5), the results show that the latter two strains harboring mutant plasmids synthesize pro-GGT.

A GGT-processing enzyme is not known yet, but GGT itself was reported to have latent protease activity (30), though it is not known if GGT is autoprocessed. Therefore, further study is needed to determine that the mutant GGTs are not processed whether because the structural changes of the mutant GGTs make it difficult for a processing-enzyme to cleave pro-GGT or because arginyl residues-513 and 571 are the active site residues for autoprocessing.

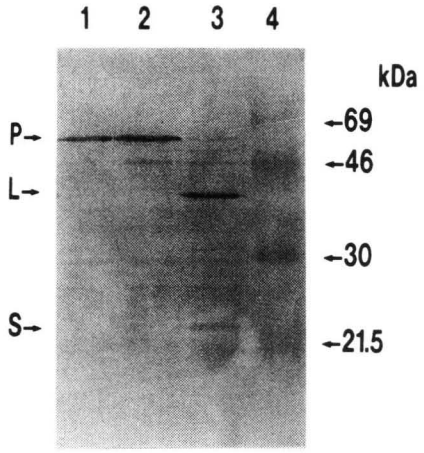

Fig. 3-3. Western blot analysis of periplasmic fractions.
Lane 1, periplasmic fraction of NS471 harboring pR571G; 2, periplasmic fraction of NS471 harboring pR513A; 3, periplasmic fraction of NS471 harboring pSH253; 4, rainbow colored protein molecular weight markers (Amersham International plc). L, S, and P indicate the positions of the large subunit, small subunit, and precursor, respectively.

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SUMMARY

Arginyl residue-114 of *Escherichia coli* K-12 γ-glutamyltranspeptidase (GGT; EC 2.3.2.2), which corresponds to arginyl residue-107 of human GGT suggested to interact with substrate, was substituted with glutamyl, lysil, glutaminyl, and histidinyl residues, respectively, by site-directed mutagenesis. Although all mutants had GGT activity, the mutant substituted with glutaminyl residue showed no difference of hydrolysis activity between with and without the acceptor. On amino acid analyses of its enzymatic reaction solutions with and without the acceptor, it had transpeptidase activity, albeit small, in comparison with wild-type GGT. These results suggest that arginyl residue-114 of *E. coli* K-12 GGT is not so important residue to interact with substrates, and that the mutant GGT substituted with glutaminyl residue may have more difficulty in binding an acceptor or the mutant GGT binding an acceptor may have more difficulty in binding a substrate than the wild-type GGT. Arginyl residues-513 and 571 of *E. coli* K-12 GGT were substituted with alanyl and glycyl residues, respectively, by oligonucleotide-directed *in vitro* mutagenesis. Both mutants were devoid of the enzymatic activity. On Western blot analysis, it was found that both mutants accumulated a GGT precursor which was not processed into large and small subunits in the periplasmic space of *E. coli* K-12.

Chapter 4. Subunit Association of γ-Glutamyltranspeptidase of *Escherichia coli* K-12

γ-Glutamyltranspeptidase (GGT) catalyzes the hydrolysis of γ-glutamyl compounds such as glutathione, and the transfer of their γ-glutamyl moieties to amino acids and peptides (1). GGT has been found in cells from bacteria to mammals, and some GGTs have been cloned (2, 3, 4, 5, 6, 7). Although the physiological roles of GGT are still controversial, GGT has been designated as the key enzyme in glutathione metabolism. GGT of *Escherichia coli* K-12 plays a role in utilization of exogenous γ-glutamyl peptides as an amino acid source (8), is localized in the periplasm (9), and is composed of one large subunit and one small subunit (10). The nucleotide sequence of its gene has been reported, and a signal peptide, large subunit, and small subunit have been shown to be encoded in a single open reading frame in this order (11). Therefore, the heterodimer is thought to be synthesized from a single precursor polypeptide through post-translational processing. The process of proteolytic cleavage was suggested to be important for correct folding of the active form by site-directed mutagenesis (12 also chapter 2, 13 also chapter 3), but the interaction of each subunit is not well understood.

Three subsites have been identified in the active center of GGT (1), one was the γ-glutamyl donor site and the others were acceptor sites. It was

assumed that the donor site was in the small subunit, and both large and small subunits participated in formation of the acceptor site. It has, however, been reported that both subunits of hepatoma GGT possessed enzymatic activity, using the separated subunits in a reversed micelle system (14), and that those of *E. coli* GGT could be separated by high-performance liquid chromatography and neither subunit alone exhibited the enzymatic activity (15).

In this chapter, the large subunit and the small subunit of *E. coli* GGT were separated by high-performance liquid chromatography and produced separately in *E. coli* cells by means of genetic engineering. The enzymatic activity and the association of both subunits were investigated.

MATERIALS AND METHODS

Materials. Restriction endonucleases, other DNA modification enzymes, sequencing primers, and polymerase chain reaction (PCR) primers were purchased from Takara Shuzo Co., Toyobo Co., and Nippon Gene Co. Anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody (from donkey), and ECL Western blotting detection reagents were purchased from Amersham International plc. Trifluoroacetic acid was purchased from Wako Pure Chemical Co. Polyvinylidene difluoride (PVDF) membranes were purchased from Nihon Millipore Kogyo K.K. A reversed-phase high-

performance liquid chromatography (HPLC) column COSMOSIL 5C18-AR-300 and acetonitrile were purchased from Nacalai Tesque Co. 2D-Silver Stain II was purchased from Daiichi Pure Chemicals Co.

Bacterial strains. The strains of *E. coli* K-12 used in this chapter are listed in Table 4-1.

Table 4-1. *E. coli* K-12 strains used in this chapter.

Strain	Genotype	Source and reference
SH641	F⁻ *ggt-2 recA56 rpsL srl300*::Tn*10*	(4)
HW459	F' *traD36 lacI*^{q} *lacZ*△*M15 proAB*$^+$/*thi rpsL endA sbcB15 hsdR4* △(*lac-proAB*) *ggt*△(*Cla*I-*Cla*I)::Kan^r *srlC300*::Tn*10 recA56*	Laboratory stock
HW460	pKKLarge/HW459	This chapter
HW470	pKKLarge and pHW185Small/HW459	This chapter
HW473	pHW185Small/HW459	This chapter
HW504	pHW71/HW459	This chapter

Growth media. LB medium containing appropriate antibiotics at 30~100 μg/ml was used for preculturing of the strains harboring recombinant plasmids. Overnight preculture of SH642, a transformant of SH641 with pSH101 (4), was subcultured at a dilution of 1:100 in LB medium and grown at 20℃ over two nights. Overnight precultures of HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), and HW504 (+GGT) were subcultured at a dilution of 1:100 in LB medium and grown at 37℃ until an absorbance at 600nm was 0.5-0.6. Then, 1mM isopropyl-β-D-

thiogalactopyranoside were added to the cultures for overexpression of the gene products. The cells were harvested after further incubation for 3 hrs.

Subcloning, transformation, and gel electrophoresis. Subcloning, transformation, and gel electrophoresis were performed as described previously (16, 17).

Preparation of periplasmic fraction. Periplasmic fraction was obtained by lysozyme treatment, as described previously (9).

Purification of GGT. GGT was purified from the periplasmic fraction of the recombinant strain SH642 to electrophoretic homogeneity by lysozyme treatment, ammonium sulfate precipitation, and chromatofocusing (4).

HPLC. Purified GGT was applied to HPLC equipped with a reversed-phase column of COSMOSIL 5C18-AR-300 (4.6 mm x 150 mm) and eluted with a mobile phase gradient of acetonitrile (0-41% in 60 min, 41-46% in 50 min, and 46-100% in 5 min) containing 0.1% trifluoroacetic acid, at a flow rate of 1.0 ml/min. A Hitachi L-6200 Intelligent Pump and a Hitachi L-4200 uv-vis detector were used. Samples were eluted at 40℃ and the absorbance at 280nm was monitored.

Mass spectrometry. The mass spectra were measured on an API III triple quadrupole mass spectrometer (Perkin Elmer Sciex) equipped with an atmospheric pressure ionization ion source (ion spray). The mass spectrometer was operated in the positive mode. The ion spray voltage was 4500V, the

interface voltage was 650V, and the orifice voltage was 90V. During analysis, the mass spectrum was scanned from 600.3 to 2001.0 amu in 0.1-amu steps. Molecular weights were calculated on the basis of deconvolution mass spectra.

Protein concentration and GGT activity determination. The protein concentration was determined by the method of Lowry *et al.* (18), with bovine serum albumin, as a standard, or by the absorbance at 280nm, assuming that the absorbance at 280nm at the concentration of 1mg protein/ml was 1.0. GGT activity was measured as described previously (9). The activity was determined with L-γ-glutamyl-*p*-nitroanilide (γ-Glu-*p*NA) as a substrate and glycylglycine (Glygly) as an acceptor. The assay solution contained 0.25μmol of γ-Glu-*p*NA, 30μmol of Glygly, 25μmol of Tris-HCl (pH8.73) and enzyme in a final volume 0.5ml. After incubation at 37℃, the absorbance at 410nm was measured.

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and native-polyacrylamide gel electrophoresis (native-PAGE) were performed as described previously (19, 20). Proteins on native-polyacrylamide gels were stained with silver using 2D-Silver Stain II according to the protocol recommended by the manufacture (Daiichi Pure Chemicals Co.). Staining for GGT activity on native-polyacrylamide gels was performed as described previously (21).

Determination of reconstituted ratio of separated subunits. The

native-polyacrylamide gel profiles obtained by staining with silver were read with a scanner, and densities of these protein bands were quantified with a Macintosh Centris 650 computer (Apple Computer, Inc.) using an NIH Image 1.54 Program. The reconstituted ratio was calculated as densities of reconstituted GGT divided by densities of separated subunits.

Western blot analysis. Proteins on SDS-polyacrylamide gels and native-polyacrylamide gels were electroblotted onto PVDF membranes (22). After blocking with TBS-T (Tris-buffered saline, pH7.6, and 0.1% Tween 20) containing 5% skimmed milk for 60 min at room temperature, the PVDF membranes were incubated with an anti-*E. coli* GGT antibody (from rabbit) for 60 min at room temperature. After the membranes had been washed with TBS-T, they were then incubated with anti-rabbit immunoglobulin, horseradish peroxidase-linked whole antibody (from donkey), for 20 min at room temperature. After washing with TBS-T, *E. coli* GGT was detected with ECL Western blotting detection reagents according to the protocol recommended by the manufacture (Amersham International plc).

DNA sequence. The correctness of the DNA sequences of the mutant plasmids was confirmed by the dideoxy-chain termination method (23, 24) with a BcaBest Dideoxy Sequencing Kit (Takara Shuzo Co.), using [α-^{32}P]dCTP (ICN Biomedicals Inc.).

PCR. PCR reaction mixtures contained 10~100ng of template DNA,

20pmol of primers and 2units of *Taq* DNA polymerase in 100μl of PCR buffer consisting of 20mM Tris-HCl (pH8.3), 1.5mM $MgCl_2$, 25mM KCl, 100μg/ml bovine serum albumin and 50μM dNTP. Each of the total of 30 cycles of PCR consisted of incubation for 30 sec at 95℃ for denaturation, 30 sec at 60℃ for annealing, and 90 sec at 72℃ for elongation. Mutagenic primers used for PCR and PCR strategy are shown in Fig. 4-1.

```
Primer-1   3'-TTCGAACGCGGAATACTCTCATTAGTTATTGACGTCCC-5'
           5'-AAGCTTGCGCCTTATGAGAGTAATCAATTACTGCAGGG-3'
              LysLeuAlaProTyrGluSerAsnGlnStop PstI
                                        codon

              Gln: C-terminal of large subunit

Primer-2   3'-AAAATCGCGGCGGCGGCGCGGAGACGTCCC-5'
           5'-TTTTAGCGCCGCCGCCGCGCCTCTGCAGGG-3'
               PheSerAlaAlaAlaAlaPro  PstI

              Ala: C-terminal of signal peptide

Primer-3   5'-CCCTGCAGACTACCCATTACTCAGTGGTGGATAAA-3'
              PstI ThrThrHisTyrSerValValAspLys

              Thr: N-terminal of small subunit

Primer-4   5'-CTGGAGAATTCCGATGATAAAACCG-3'
                 EcoRI   Initiation
                         codon
```

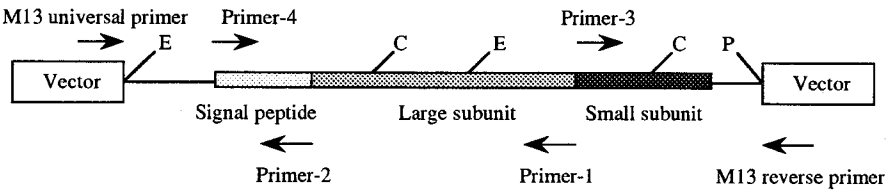

Fig. 4-1. Mutagenic primers used in PCR and PCR strategy for construction of plasmids for separate expression of large subunit and small subunit.
E, *Eco*RI; C, *Cla*I; P, *Pst*I.

Construction of plasmid pKKLarge. The plasmid pKKLarge encoding only the signal peptide and the large subunit just downstream of *tac* promoter was constructed as follows. PCR was performed between M13 universal primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and primer-1 introducing a stop codon in place of threonyl residue-391, the N-terminal amino acid of the small subunit, and the following *Pst*I site, using pSH253 (25) as a template. The PCR product was cleaved with *Cla*I and *Pst*I. Plasmid pHW71 (13) containing the full length *ggt* just downstream of the *tac* promoter was cleaved with *Cla*I and *Pst*I. The *Cla*I-*Pst*I fragment (0.8kb) of the PCR product was ligated with the larger fragment (4.9kb) of pHW71 cleaved with *Cla*I and *Pst*I, to produce the plasmid pKKLarge (5.7kb).

Construction of plasmid pKKSmall. PCR was performed between M13 universal primer and primer-2 introducing the *Pst*I site just downstream of the DNA encoding the signal peptide using pSH253 as a template. The PCR product was cleaved with *Eco*RI and *Pst*I, and the *Eco*RI-*Pst*I fragment (1.2kb) was ligated with the larger fragment (3.1kb) of pSH253 cleaved with *Eco*RI and *Pst*I, to produce the plasmid pGGTSignal (4.4kb) encoding only the signal peptide. PCR was also performed between M13 reverse primer (5'-AGCGGATAACAATTTCACACAGGAAAC-3') and primer-3 introducing the *Pst*I site just upstream of the gene encoding the small subunit, using pSH253 as a template. The PCR product was cleaved with *Pst*I and the *Pst*I fragment

(0.8kb) was ligated with pGGTSignal (4.4kb) cleaved with *Pst*I, to produce the plasmid pGGTSmall (5.2kb) encoding only the signal peptide and the small subunit. Then, the plasmid pKKSmall encoding the signal peptide and the small subunit just downstream of the *tac* promoter was constructed as follows. PCR was performed between M13 reverse primer and primer-4 introducing an *Eco*RI site just upstream of the initiation codon of *ggt*, and using pGGTSmall as a template. The PCR product was cleaved with *Eco*RI and *Hin*dIII, and the *Eco*RI-*Hin*dIII fragment (0.9kb) was ligated with the larger fragment (4.6kb) of plasmid pKK223-3 (26) also cleaved with *Eco*RI and *Hin*dIII, to produce pKKSmall (5.5kb).

Construction of plasmid pHW185Small. Plasmid pACYC184 (27) carries the origin of replication from plasmid p15A, which is compatible with vectors such as pKK223-3 which carries the ColE1 origin. pKKSmall was cleaved with *Bam*HI and *Dra*I. The smaller *Bam*HI-*Dra*I fragment (1.9kb) of pKKSmall containing the DNA encoding the signal peptide and small subunit in that order between the *tac* promoter and the ribosomal terminator, was ligated with pACYC184 cleaved with *Bam*HI and *Eco*RV (4.1kb), to produce the plasmid pHW185Small (6.0kb).

Determination of specific activity on Western blot analysis. The Western blot profiles were read with a scanner, and densities of these protein bands were quantified with a Macintosh Centris 650 computer (Apple

Computer, Inc.) using an NIH Image 1.54 Program. Specific activity was calculated as activity divided by densities.

RESULTS

Separation of subunits of *E. coli* GGT by HPLC. Purified GGT (855μg) was treated by HPLC as described under Materials and Methods (Fig. 4-2). As shown in Fig. 4-2, peak-1 and peak-2 were eluted at 70 min and 88 min, respectively. These peaks were pooled, respectively, dialyzed against 2mM Tris-HCl buffer (pH8.3) and concentrated in a centrifugal concentrator. Pooled peak-1, peak-2, and purified GGT were subjected to SDS-PAGE (Fig. 4-2). As shown in Fig. 4-2, peak-1 showed the same mobility as the large subunit and peak-2 as the small subunit of purified GGT, indicating these peaks were the large subunit and the small subunit, respectively.

Mass spectrometry of peak-1 and peak-2. The ion spray mass spectra of peak-1 and peak-2, and the deconvolution mass spectra obtained by data processing from the spectra are shown in Fig. 4-3. The molecular weights of peak-1 and peak-2 were determined to be 39,207 and 20,015, respectively.

GGT activity of separated subunits and their mixture. The large subunit (49μg) exhibited no GGT activity and the small subunit (45μg) had the

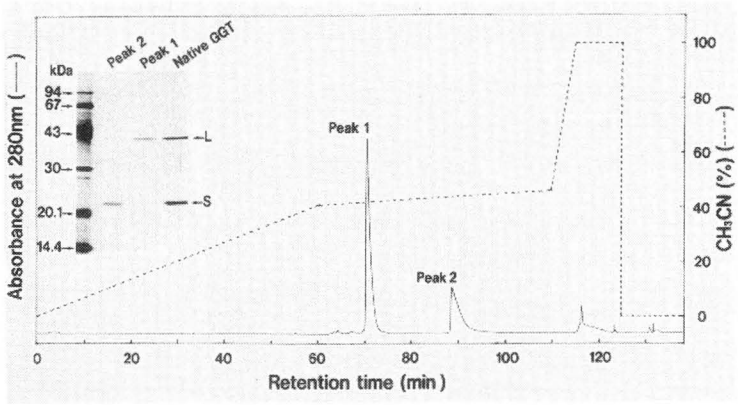

Fig. 4-2. HPLC profile of purified GGT with reversed-phase HPLC column COSMOSIL 5C18-AR-300 and SDS-PAGE analysis of peak-1, peak-2, and purified GGT.
HPLC profile; peak-1 and peak-2 were eluted at retention times of 70 min and 88 min, respectively. The solid line shows absorbance at 280nm and the broken line shows concentration (%) of solvent acetonitrile. SDS-PAGE; a portion of peak-1 and peak-2 separated by reversed-phase HPLC, and purified native GGT were subjected to SDS-PAGE, followed by silver staining. L and S indicate the positions of the large subunit and small subunit, respectively.

enzymatic activity of 0.0029munits. However, following preincubation at 4℃ for 15hrs in 20mM Tris-HCl buffer pH8.3, a mixture of the large (49μg) and small (45μg) subunits at 4℃ exhibited the enzymatic activity of 0.031munits, albeit only partial. To increase the enzymatic activity of the mixture, the mixture of the large (49μg) and small (45μg) subunits was preincubated at 37

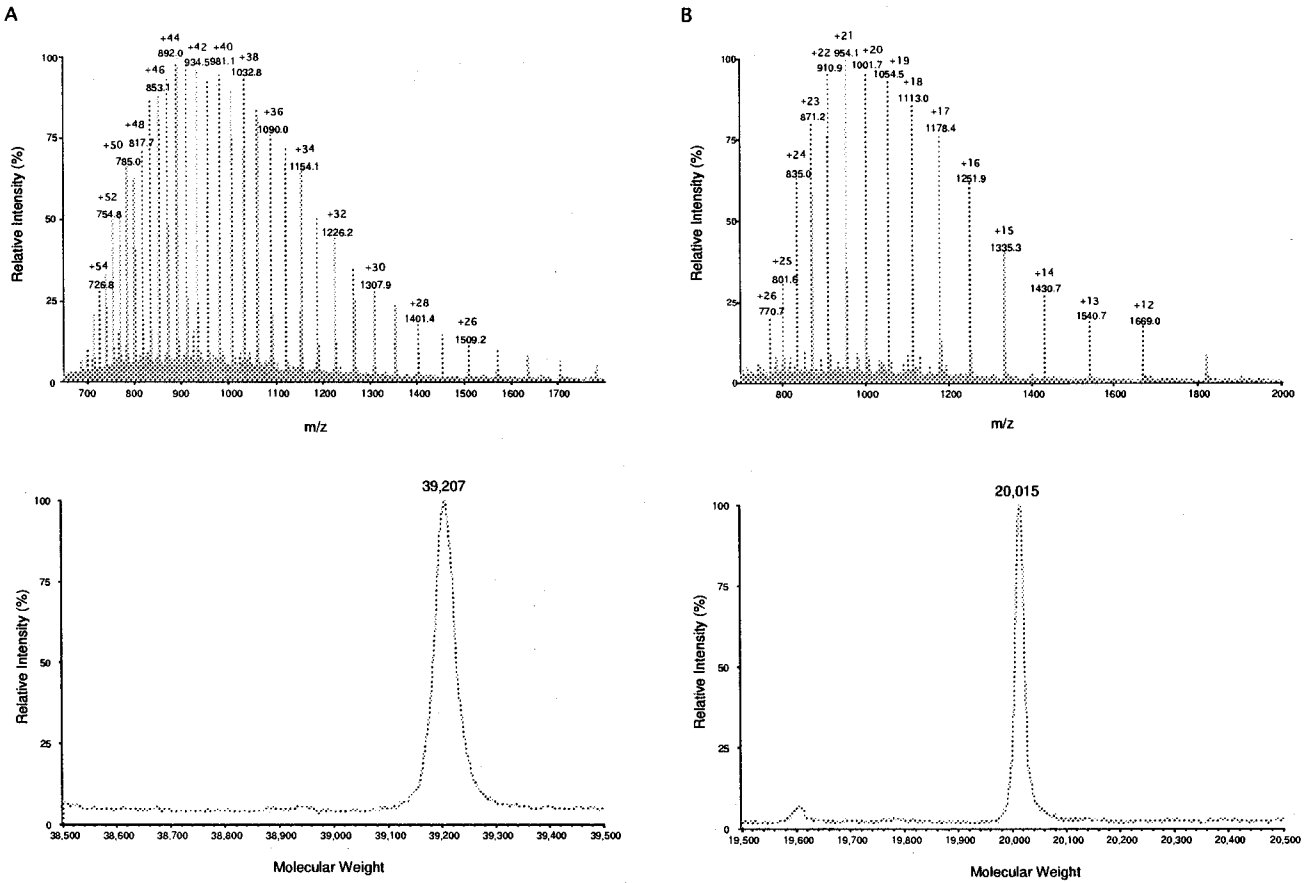

Fig. 4-3. Ion spray mass spectra of peak-1 and peak-2. The ion spray mass spectrometry of peak-1 and peak-2 separated by reversed-phase HPLC was performed. Upper mass spectra are ion spray mass spectra. The numbers above the peaks are the m/z and the predicted charges. Lower mass spectra are deconvolution mass spectra. A, peak-1; B, peak-2.

℃ for 15hrs in 20mM Tris-HCl buffer pH8.3. The enzymatic activity of the mixture was 0.06munits.

Native-PAGE of separated subunits and their mixture. The same samples as those of which GGT activity was measured, i. e. the separated large subunit, small subunit, and mixtures of both, were subjected to native-PAGE to investigate the reassociation of the separated subunits. Proteins on the gel were stained with silver (Fig. 4-4A). The small subunit (A, lane 3), large subunit (A, lane 4), and purified native GGT (A, lane 5) showed different mobilities. Although most of the large subunit and small subunit remained, new bands which had the same mobility as the purified native GGT appeared in mixtures of separated subunits at 37℃ (A, lane 1) and 4℃ (A, lane 2). The reconstituted ratio of separated subunits in the mixture at 4℃ was approximately 4%. Staining of the gel for GGT activity was also carried out (Fig. 4-4B). While the small subunit (B, lane 3) and the large subunit (B, lane 4) alone showed no activity band, red bands indicating GGT activity were observed in mixtures of separated subunits at 37℃ (B, lane 1) and 4℃ (B, lane 2) which showed the same mobility as purified native GGT.

Separate expression of the large subunit and the small subunit. Separate expression of each subunit was attempted to investigate whether the separately expressed large subunit and small subunit or their mixture exhibit GGT activity or not. The plasmid pKKLarge encoding only the signal peptide

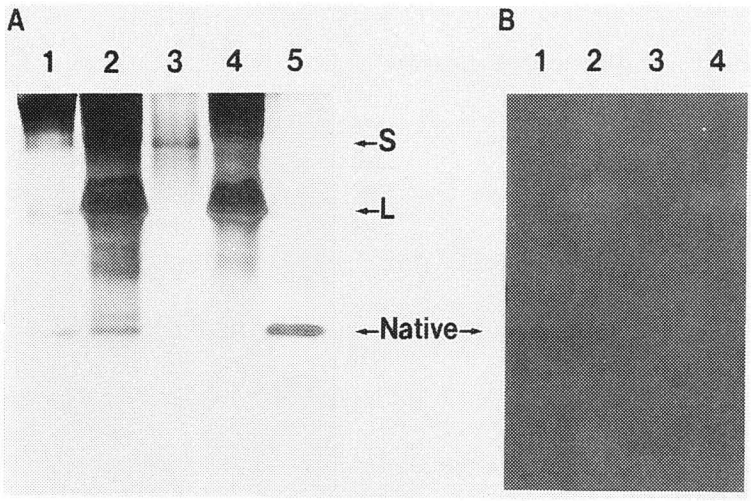

Fig. 4-4. Native-PAGE analysis of separated subunits and their mixture.
Separated subunits and purified GGT were subjected to native-PAGE, followed by silver staining (A) and staining for GGT activity (B). A; lane 1, the mixture of separated small (45μg) and large (49μg) subunits at 37℃; lane 2, the mixture of separated small (45μg) and large (49μg) subunits at 4℃; lane 3, small subunit (45μg); lane 4, large subunit (49μg); lane 5, the purified native GGT. Native, S, and L indicate the position of the purified native GGT, small subunit, and large subunit, respectively. B; lane 1, the mixture of separated small (45μg) and large (49μg) subunits at 37℃; lane 2, the mixture of separated small (45μg) and large (49μg) subunits at 4℃; lane 3, small subunit (45μg); lane 4, large subunit (49μg). Native indicates the position of the purified native GGT.

and the large subunit just downstream of the *tac* promoter of pKK223-3, and the plasmid pKKSmall encoding only the signal peptide and the small subunit just downstream of the *tac* promoter of pKK223-3 were constructed, as described under Materials and Methods (Fig. 4-5). The expected mutations of

ggt in pKKLarge and pKKSmall were confirmed by DNA sequencing. The GGT-deficient strain HW459 (-GGT) was transformed with pKKLarge or pKKSmall, transformants were named HW460 (+Large) and HW461, respectively. Cell lysates of HW460 (+Large) and HW461 were subjected to SDS-PAGE followed by Western blot analysis and shown to produce only the large subunit and the small subunit, respectively (data not shown). To examine the interaction of large and small subunits on their co-expression, the plasmid pHW185Small, compatible with pKKLarge, was constructed as described under Materials and Methods (Fig. 4-5). HW459 (-GGT) and HW460 (+Large) were transformed with pHW185Small, and the transformants were named HW473 (+Small) and HW470 (+Large, +Small), respectively. HW459 (-GGT) was transformed with the plasmid pHW71 containing the entire GGT structural gene just downstream of the *tac* promoter of pKK223-3, and the transformant was named HW504 (+GGT). Cell lysates of HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), and HW504 (+GGT) were subjected to SDS-PAGE, followed by Western blot analysis. HW460 (+Large) produced only the large subunit, HW473 (+Small) produced only the small subunit, and HW470 (+Large, +Small) and HW504 (+GGT) produced both subunits (data not shown).

Localization of the large subunit and the small subunit produced by the constructed plasmids. The periplasmic fractions were obtained

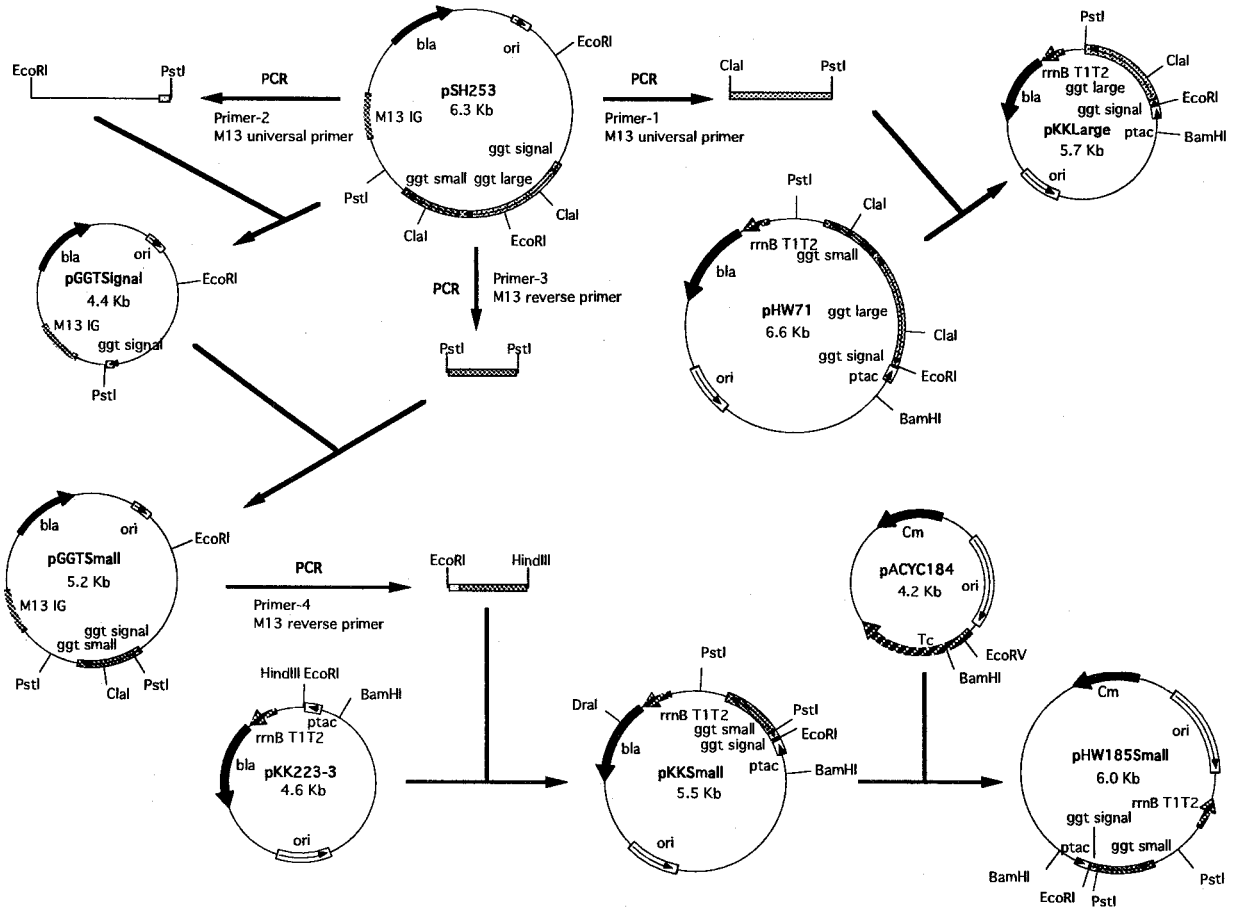

Fig. 4-5. Construction of plasmids for separate expression of large and small subunits of GGT. The strategy for construction of plasmids is described in detail under Materials and Methods. ori, replication origin; ptac, *tac* promoter; rrnBT1T2, ribosomal terminator; M13IG, intergenic region of M13 phage DNA; bla, ampicillin resistance; Tc, tetracycline resistance; Cm, chloramphenicol resistance.

from HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), and HW504 (+GGT), and a total of 20μg of protein of each periplasmic fraction was subjected to SDS-PAGE, followed by Western blot analysis (Fig. 4-6). The periplasmic fraction of HW460 (+Large) (lane 2) contained only the large subunit, that of HW473 (+Small) (lane 4) contained only the small amount of small subunit, and those of HW470 (+Large, +Small) (lane 3) and HW504 (+GGT) (lane 5) contained both subunits. The expression level of the large subunit of HW460 (+Large) was 40% of that of

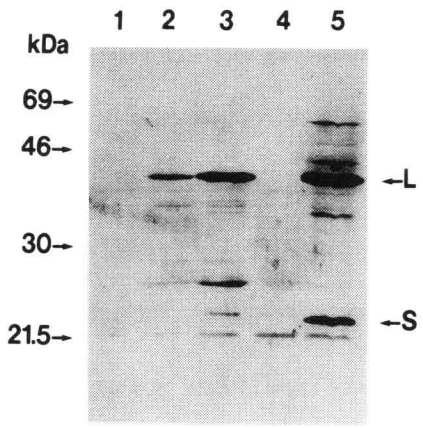

Fig. 4-6. Western blot analysis of periplasmic fractions of recombinant strains. I. Periplasmic fractions (20μg of protein) of HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), and HW504 (+GGT) were subjected to SDS-PAGE, followed by Western blot analysis. Lane 1, HW459 (-GGT); lane 2, HW460 (+Large); lane 3, HW470 (+Large, +Small); lane 4, HW473 (+Small); lane 5, HW504 (+GGT). L and S indicate the positions of the large subunit and small subunit, respectively.

HW504 (+GGT) and the expression level of the small subunit of HW473 (+Small) was 7.3% of that of HW504 (+GGT). The profiles on Western blot analysis of periplasmic fractions were very similar to those of cell lysates. No GGT was detected in cytoplasmic or membrane fractions (data not shown). Therefore, the mutant gene product of each strain was located in the periplasmic space.

GGT activities of HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), and HW504 (+GGT). The GGT activities of the periplasmic fractions of HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), and HW504 (+GGT) were measured (Table 4-2). HW459 (-GGT), HW460 (+Large), and HW473 (+Small) exhibited no enzymatic activity. HW470 (+Large, +Small) and

Table 4-2. GGT activity of periplasmic fraction from each strain.

Strains	Protein (mg)	Total activity (mu)	Specific activity (mu/mg)
HW459 (-GGT)	10.2	$<1x10^{-2}$	—
HW460 (+Large)	10.2	$<1x10^{-2}$	—
HW470 (+Large, +Small)	12.1	3.28	0.271
HW473 (+Small)	11.1	$<1x10^{-2}$	—
HW504 (+GGT)	8.04	3.35×10^{2}	41.8

Each strain was cultured in 100ml of LB medium as described under Materials and Methods. Periplasmic fraction of each strain was prepared by lysozyme treatment, GGT activity of periplasmic fraction of each strain was measured with γ-Glu-*p*NA as a substrate and Glygly as an acceptor, and its protein concentration was determined by the method of Lowry *et al.*

HW504 (+GGT) exhibited GGT activity, however, GGT activity of HW470 (+Large, +Small) was much lower than that of HW504 (+GGT). When whole-cell GGT activity of each strain was measured instead of just that in the periplasmic fraction, the similar results were obtained (data not shown).

Native-PAGE of periplasmic fractions of HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), and HW504 (+GGT). Periplasmic fractions of HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), and HW504 (+GGT), and purified native GGT were subjected to native-PAGE, followed by Western blot analysis (Fig. 4-7). HW470 (+Large, +Small) and HW504 (+GGT) showed bands with the same mobility as the purified native GGT on the gel and were also recognized by anti-*E. coli* GGT antibody. From the rough comparison of specific activity of periplasmic fractions of HW470 (+Large, +Small) and HW504 (+GGT), the specific activity of the periplasmic fraction of HW470 (+Large, +Small) was approximately one fortieth of that of HW504 (+GGT). No GGT was detected in the periplasmic fractions of HW459 (-GGT), HW460 (+Large), and HW473 (+Small).

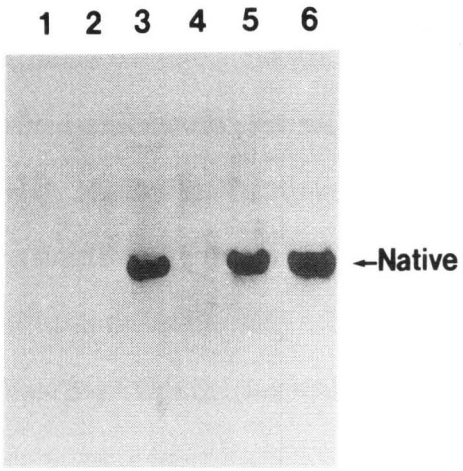

Fig. 4-7. Western blot analysis of periplasmic fractions of recombinant strains. II. Periplasmic fractions (30μg of protein) of HW459 (-GGT), HW460 (+Large), HW470 (+Large, +Small), HW473 (+Small), HW504 (+GGT), and 1.5μg of purified native GGT were subjected to native-PAGE, followed by Western blot analysis. Lane 1, HW459 (-GGT); lane 2, HW460 (+Large); lane 3, HW470 (+Large, +Small); lane 4, HW473 (+Small); lane 5, HW504 (+GGT); lane 6, purified native GGT.

DISCUSSION

As reported previously (15), the large subunit and the small subunit of *E. coli* GGT can be separated by HPLC. The molecular weights of both subunits were almost identical to those calculated from DNA sequence data (39,198 and 20,010, respectively).

Though a little damage might be done to the separated subunits during HPLC step, the large subunit separated by HPLC exhibited no GGT activity and the small subunit exhibit scarcely detectable activity, the mixture of both subunits showed partial recovery of activity. This result suggests that the separated large and small subunits could be reconstituted and the reconstituted exhibited the enzymatic activity. However, the activity of the mixture was very low in comparison with the native GGT (6mu/μg). It could not be confirmed whether the slight activity of the separated small subunit might be due to the activity of the small subunit by itself or the reconstituted GGT because of the very slight contamination of the separated large subunit. From the results of native-PAGE (Fig. 4-4A, B), some of the separated subunits appear to associate and be folded back into heterodimeric form as native GGT, and only the correctly folded form expresses the enzymatic activity. A lot amount of the heterogeneous composition of the large subunit and the small subunit observed on native-PAGE (Fig. 4-4A) may have been due to unfolding and/or instability of the molecules when they did not associate with each other. The decrease of separated subunits in the mixture at 37℃ may have been caused by a possible latent proteinase activity of the separated small subunit. In fact, there was a report on latent proteinase activity of the small subunit of rat GGT (28).

In this chapter, the separate expression of the large and small subunits of *E. coli* GGT was achieved by genetic engineering for the first time. This

method will facilitate in future construction of chimeric GGTs comprised of large subunits derived from bacteria and small subunits from mammalian.

Each of the subunits expressed from recombinant plasmids was secreted into the periplasmic space. In Fig. 4-6, all strains gave a band corresponding to a molecular weight of 21.5kDa, and HW460 (+Large) and HW470 (+Large, +Small) possessed a protein of 26kDa. The protein of 21.5kDa might be derived from the destructed *ggt* of HW459 (-GGT), and the protein of 26kDa from the large subunit by abnormal proteolysis. The reason why the amount of independently expressed subunits were small seemed to be caused by their susceptibility to proteolytic enzymes, probably because of their unfolding or incorrect folding. The strain producing either the large subunit or the small subunit exhibited no enzymatic activity, however, when both were co-produced in the same cell from the independent plasmid, GGT activity was partially recovered. This result is almost compatible with the result of separated subunits by HPLC. Native-PAGE followed by Western blot analysis (Fig. 4-7) suggested that the large subunit and the small subunit expressed independently can associate and be folded into active structure as native GGT. All GGTs, which have been cloned from bacteria to mammals, are known to be synthsized from a single mRNA. So, it is very interesting that the mutant GGT consisting of the expressed large nad small subunits independently, exhibits the enzymatic activity, *in vivo*. Interestingly, they seemed only to be

folded after they were secreted into the periplasmic space because no GGT protein and activity could be detected in the cytoplasmic fraction and the membrane fraction. The reason why no large or small subunit was detected in the periplasmic fractions of HW460 (+Large) or HW473 (+Small) on native-polyacrylamide gel may be caused by the heterogeneous composition of the large subunit and small subunit produced alone, which might be not correctly folded or unfolded in the cells.

The specific activity of the periplasmic fraction of HW470 (+Large, +Small) (+Large, +Small) was much lower than that of HW504 (+GGT) (+GGT). This indicates that the expressed large and small subunits may be difficult to associate and/or be folded in a suitable conformation, and that only a portion of the associated molecules can be fold into an active structure. Though we cannot deny the possibility that the remaining peptide of the added signal peptide artificially at the N-terminal of the small subunit disturbs the association.

There were previous reports that the propeptide of rat kidney GGT exhibited less than 2% of transpeptidase activity shown by the dimeric enzyme (29) and that each of the separated subunit of rat kidney GGT by SDS and urea exhibited no enzymatic activity (30). We are successful to obtain mutant precursors of *E. coli* GGT which have a replaced amino acid residues at the processing site between the large and small subunits and become to be

processed only partially, although the mutant precursors exhibited no enzymatic activity, once they were processed, they came to exhibit GGT activity (13). These previous reports and the results of this chapter suggest that the active site resides in the interface between large and small subunits, and that the processing of the precursor into heterodimeric form is necessary for formation of the active center. The correct association and folding of both subunits are probably necessary to construct proper structure of active site and to express the enzymatic activity. The previous synthesis of GGT in its precursor form and the successive processing seems to be the necessary steps to retain the most effective way to construct intact structure of GGT.

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SUMMARY

γ-Glutamyltranspeptidase (GGT, EC: 2.3.2.2) of *Escherichia coli* K-12 consists of one large subunit and one small subunit, the two of which can be separated by high-performance liquid chromatography. The large subunit exhibited no GGT activity and the small subunit had little enzymatic activity, however, a mixture of both subunits showed partial recovery of the enzymatic activity. From the results of native-polyacrylamide gel electrophoresis, it was suggested that they could partially recombine, and that recombined dimer exhibited the enzymatic activity. The large subunit and the small subunit were expressed independently by genetic engineering. Although the strains producing each subunit alone exhibited no GGT activity, the strain producing both subunits exhibited the enzymatic activity. However, the specific activity of the strain was approximate 3% of that of a strain harboring a plasmid encoding the intact structural gene. These results indicate that a portion of the large subunit and the small subunit can associate *in vitro* and *in vivo*, and that a part of the associated subunits is able to be folded into an active structure, and suggest that the correct association and folding of both subunits are probably necessary to construct proper structure of active site and to express the enzymatic activity.

Conclusions

The author performed studies on γ-glutamyltranspeptidase (GGT, EC: 2.3.2.2) of *Escherichia coli* K-12 to elucidate (1) the mechanism of the expression of GGT induced at a low temperature, (2) the mechanism of processing to make large and small subunits, (3) the amino acid residues acting as active sites, and (4) the interaction of two subunits and expression of the activity.

The findings in each chapter are summarized as follows:

Chapter 1. Low Temperature Inducible γ-Glutamyltranspeptidase of *Escherichia coli* K-12

Escherichia coli K-12 cultured at 20℃ exhibits higher GGT activity than that cultured at 37℃ or 42℃. GGT of *E. coli* K-12 is synthesized from a single precursor polypeptide into heterodimeric form by post-translational processing. On Western blot analysis, *E. coli* K-12 cells cultured at 20℃ produced more GGT protein than those cultured at 37℃. *E. coli* K-12 cells were cultured at 20℃, 37℃, and 42℃, and mRNA of GGT gene (*ggt*) in the cells were quantified, respectively, by Northern blot analysis. The level of *ggt* mRNA at 20℃ was 10-fold higher than that at 37℃. These findings show that the higher GGT activity in *E. coli* K-12 cells grown at 20℃ was due to a

higher expression level of GGT protein at 20℃ caused by a higher level of *ggt* mRNA at 20℃. The promoter region of *E. coli* K-12 *ggt* was identified by primer extension analysis and by deletion of the 5'-region of *ggt*. A fusion gene composed of *ggt* promoter and *lacZ* was constructed. The lower growth temperature had a slight effected on β-galactosidase activity expressed from the fusion gene. The *ggt* mRNA at 20℃ was found to be more stable than that at 37℃. This suggests that the higher level of *ggt* mRNA at 20℃ was affected by a low temperature dependent *ggt* promoter as well as the stability of *ggt* mRNA at 20℃.

Chapter 2. Effect of Site-Directed Mutations on Processing and Activity of γ-Glutamyltranspeptidase of *Escherichia coli* K-12

Cells of a GGT-overproducing transformant of *E. coli* K-12 were fractionated and the localization of the enzyme was examined by Western blot analysis. The periplasmic fraction only contained the mature form of GGT, the membrane fraction only contained the precursor of GGT, and no precursor of GGT was detected in the cytoplasmic fraction. Amino acid residues at the cleavage site for processing into the large and small subunits were substituted by site-directed mutagenesis. The processing phenotypes of six mutants were examined by Western blot analysis, and their GGT activities were measured. Mutations at the N-terminal amino acid residues of the small subunit (Thr-391,

Thr-392, and His-393) prevented the maturation of the enzyme and the immature mutants exhibited no enzymatic activity. A mutation at the C-terminal residue of the large subunit (Gln-390) had less effect on the processing and enzymatic activity. These findings suggest that the sequence of threonyl-threonyl-histidinyl residues at the N-terminal of the small subunit is very important for the processing of *E. coli* K-12 GGT and this processing is essential to the expression of GGT activity of *E. coli* K-12.

Chapter 3. Site-Directed Mutations at Arg-114, 513, and 571 of γ-Glutamyltranspeptidase of *Escherichia coli* K-12

Arginyl residue-114 of *E. coli* K-12 GGT, which corresponds to arginyl residue-107 of human GGT suggested to interact with substrate, was substituted with glutamyl, lysil, glutaminyl, and histidinyl residues, respectively, by site-directed mutagenesis. All mutants had the hydrolysis activity of the γ-glutamyl compound. Three mutants except the mutant substituted with glutaminyl residue, and wild-type GGT exhibited higher hydrolysis activity in the presence of an acceptor than in the absence of the acceptor, although the differences in the activity of the mutant GGT between with and without the acceptor were smaller than that of the wild-type GGT, while the mutant GGT substituted with glutaminyl residue showed no difference in hydrolysis activity with and without the acceptor. The amino acid

analyses of the enzymatic reaction solutions of the mutant GGT with and without the acceptor revealed the presence of transpeptidase activity, albeit small, in comparison with the wild-type GGT. These findings suggest that the arginyl residue-114 of *E. coli* K-12 GGT is not so important to bind substrates, and that the mutant GGT substituted with glutaminyl residue may have more difficulty in binding an acceptor or the mutant GGT binding an acceptor may have more difficulty in binding a substrate than the wild-type GGT. Arginyl residues-513 and 571 of *E. coli* K-12 GGT were substituted with alanyl and glycyl residues, respectively, by oligonucleotide-directed *in vitro* mutagenesis. Both mutants were devoid of the enzymatic activity. Western blot analysis revealed that both mutants accumulated a GGT precursor which was not processed into large and small subunits in the periplasmic space of *E. coli* K-12.

Chapter 4. Subunit Association of γ-Glutamyltranspeptidase of *Escherichia coli* K-12

Large and small subunits of *E. coli* K-12 GGT can be separated by high-performance liquid chromatography. Using ion spray mass spectrometry, the masses of the large and small subunits were determined to be 39,207 and 20,015, respectively. The large subunit exhibited no GGT activity and the small subunit had little enzymatic activity, but, a mixture of both subunits

showed partial recovery of the enzymatic activity. The results of native-polyacrylamide gel electrophoresis suggested that they were partially recombined, and that the recombined dimer exhibited enzymatic activity. The gene of the GGT encoded signal peptide, large and small subunits in the single open reading frame in this order. Two kinds of plasmids were constructed encoding signal peptide and either the large or small subunit. A GGT-less mutant of *E. coli* K-12 was transformed with each plasmid or with both of them. The strain harboring the plasmid encoding each subunit produced a small amount of the corresponding subunit protein in the periplasmic space, but exhibited no enzymatic activity. The strain transformed with both plasmids together exhibited the enzymatic activity, but, the specific activity of the strain was approximately 3% of that of the strain harboring a plasmid encoding the intact structural gene. These findings indicate that a portion of the separated large and small subunits can be reconstituted *in vitro* to exhibit the enzymatic activity, and that the expressed large and small subunits independently are able to associate *in vivo* and be folded into an active structure although the specific activity of the associated subunits was much lower than that of the native enzyme. This suggests that the synthesis of γ-glutamyltranspeptidase in a single precursor polypeptide and the subsequent processing are more effective to construct the intact structure of γ-glutamyltranspeptidase than the association of the separated large and small subunits.

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List of Publications

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(2) Hashimoto, W., Suzuki, H., Yamamoto, K., and Kumagai, H. (1995) Effect of site-directed mutations on processing and activity of γ-glutamyltranspeptidase of *Escherichia coli* K-12. *J. Biochem.* **118**, 75-80

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(4) Hashimoto, W., Suzuki, H., Nohara, S., Tachi, H., Yamamoto, K., and Kumagai, H. Subunit association of γ-glutamyltranspeptidase of *Escherichia coli* K-12. *J. Biochem.* in press.

(5) Suzuki, H., Hashimoto, W., Nakayasu, T., Yamamoto, K., and Kumagai, H. Mutational analysis of *Escherichia coli* K-12 γ-glutamyltranspeptidase by site-directed mutagenesis. in preparation.

Related issue

(6) Kumagai, H., Nohara, S., Suzuki, H., Hashimoto, W., Yamamoto, K., Sakai, H., Sakabe, K., Fukuyama, K., and Sakabe, N. (1993) Crystallization and preliminary X-ray analysis of γ-glutamyltranspeptidase from *Escherichia coli* K-12. *J. Mol. Biol.* **234**, 1259-1262